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EXPERIMENTAL INFECTIVE PNEUMOCONIOSIS. II: COAL-MINE DUST WITH ATTENUATED TUBERCLE BACILLI (B.C.G.) IN THE LUNGS OF IMMUNISED GUINEA-PIGS.

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THE prevalence of chronic pulmonary disease among coal-miners has long been recognised, but the relationship of tuberculosis and coal-miners' pneumoconiosis has remained in dispute. Cox (1857) observed a high incidence of "tubercular consumption" among miners in South Lancashire, and suggested that coal dust predisposed to tuberculosis; Greenhow (1862) and Oliver (1909) recognised pulmonary phthisis among colliers. But many workers considered that coal-mining was not injurious to health and that coal-mine dust was protective against tuberculosis (Tatham, 1902; Wainwright and Nichols, 1905; Haythorn, 1913; Haldane, 1916, 1918). Mavrogordato (1922) stated "It is well known that colliers do not now develop miners' phthisis", and Haldane (1923) "that the dust they breathe protects them from serious dangers".

The clinical studies of Boehme (1925) in coal-miners of the Ruhr and those of Tattersall (1926) in South Wales miners showed a high incidence of tuberculosis to be a frequent terminal feature. Kettle (1930, 1932, 1934) suggested that there was a close relationship between tuberculosis and coal-miners' pneumoconiosis. Cummins and Sladden (1930) and Cummins (1931, 1934, 1935, 1936) considered that tuberculosis occurred frequently among coal-miners. Sokoloff (1936) examined 418 anthracite coal-miners and demonstrated tubercle bacilli in 203 of them. Sen (1937) found tubercle bacilli in the sputa of 12 of 100 cases of coal-miners' pneumoconiosis. Belt and Ferris's (1942) suggestion that tuberculosis occurs more frequently among coal-miners than had been supposed, though it is often difficult to recognise, has been supported by recent pathological and clinical studies (Cummins, 1940; Rogers, 1946; Gough, 1947; Fletcher, 1948; Worth and Dickman, 1950; van Mechelen, 1951; James, 1954; Fletcher, 1955; von Gerstel, 1954).

In previous work in this laboratory (Attygalle, Harrison, King and Mohanty, 1954) rats and guinea-pigs were injected intratracheally with a mixture of coal-mine dust and killed B.C.G. organisms. Although combined lesions were formed containing collagen fibres, a result that did not occur in the animals given either

dust or bacilli alone, the extent of the fibrogenic reaction was slight. In this present experiment it was hoped to increase the intensity of the tuberculous stimulus by employing allergic animals and by using living tubercle bacilli capable of setting up a chronic though non-fatal infection. In addition, to simulate conditions in man, it was considered necessary that infection should reach the lungs through the trachea and that the amount of coal-mine dust introduced into the lungs should be comparable on a weight basis to that found in coal-miners. We have therefore injected B.C.G.-immunised guinea-pigs by the intratracheal route with a mixture of living bacilli of the attenuated B.C.G. strain intimately mixed with 100 mg. of coal-mine dust for each animal, on the assumption that this amount is comparable with the average of about 40 g. of coal-dust found in the lungs of cases of progressive massive fibrosis (King and Nagelschmidt, 1955).

MATERIALS AND METHODS.

Tubercle bacilli.

The B.C.G. strain employed was a sub-culture obtained from Dr. Holt, Central Public Health Laboratory, Colindale, London, of the standard strain used for vaccination in Great Britain, prepared at the State Serum Institute, Copenhagen. The suspension for inoculating the guinea-pigs was prepared from a 14-day culture on Löwenstein-Jensen medium. A weighed mass of bacilli, scraped from the surface of the slopes, was shaken with 0·85 per cent saline in a bottle containing glass beads for 5 min. on an electric shaker. Suitable dilutions of the suspension were made in saline.

Coal-mine dust.

Since it is difficult to obtain a sufficiently large sample of air-borne coal-mine dust with particles of respirable size, an artificial mixture was prepared, to resemble in chemical and mineralogical composition air-borne dusts in South Wales collieries. Shale is the rock accompanying the coal seam and provides the siliceous material in the dust. Anthracite coal dust 4·8 g. and shale dust (Ammonford B) 1·2 g. were mixed together gradually in small quantities on glazed paper, and the mixture was ground overnight in an electrically driven mortar. The compositions of these dusts are shown in the Table. Suspensions of dust in saline were autoclaved, 1 per cent of sterile guinea-pig serum was added to keep the dust well suspended, and the mixtures were agitated until the moment of use in an electric shaking machine.

EXPLANATION OF PLATES.

FIG. 1.—Guinea-pig lung 30 days after intratracheal injection of 100 mg. coal-mine dust. Coal particles are seen lying in small aggregates in alveoli. (H.E. × 50.)

FIG. 2.—300 days after intratracheal injection of 100 mg. coal-mine dust. Coal-dust is collected in definite foci. The dust particles are held together at places by a few fine reticulin fibres. (H.E. × 50.)

FIG. 3.—30 days after intratracheal injection of 10 μg. living B.C.G. Thickened alveolar walls due to cellular proliferation obliterating some of the alveoli. (H.E. × 175.)

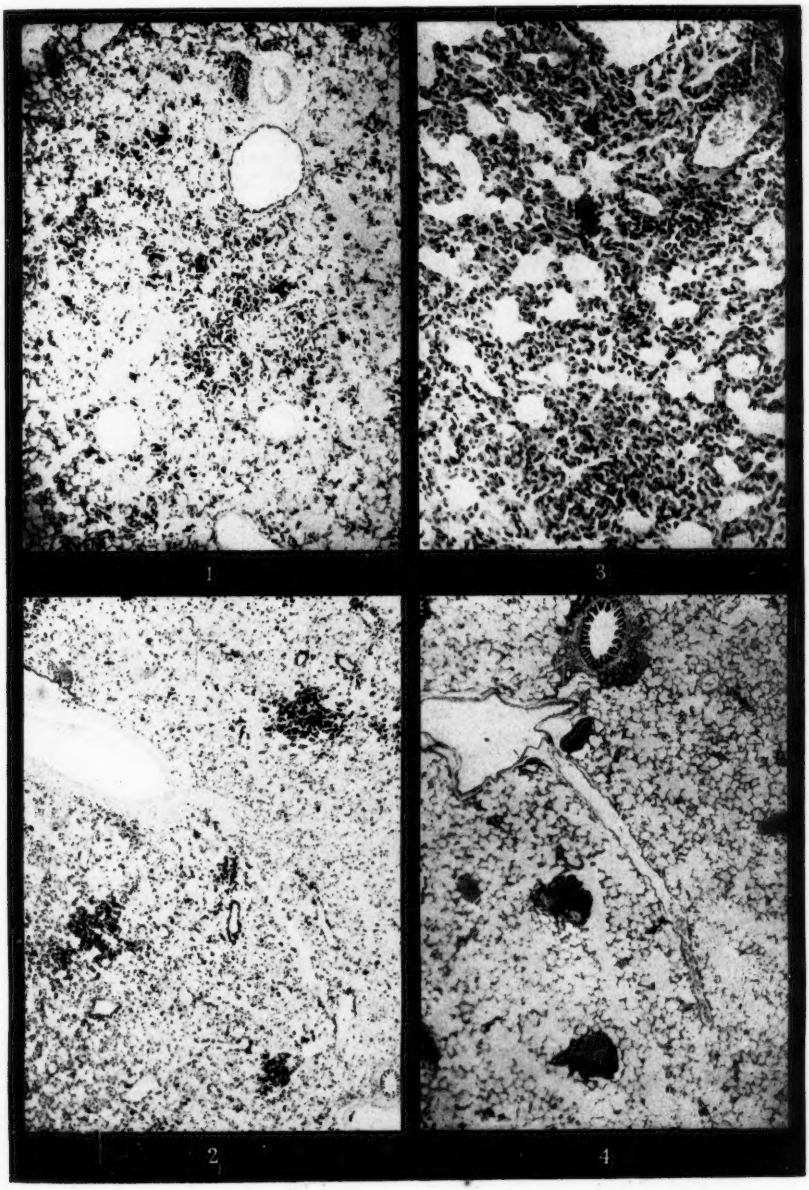
FIG. 4.—365 days after intratracheal injection of 10 μg. living B.C.G. Normal lung parenchyma but some occasional lymphoid hyperplasia. (H.E. × 40.)

FIG. 5.—Guinea-pig lung 30 days after intratracheal injection of 10 μg. living B.C.G. and 100 mg. coal-mine dust. Coal particles lying in aggregates in alveoli. An area of cellular proliferation showing fine dust particles. (H.E. × 175.)

FIG. 6.—60 days after intratracheal injection of 10 μg. living B.C.G. and 100 mg. coal-mine dust. Large dust aggregates in proliferated area. (H.E. × 175.)

FIG. 7.—120 days after intratracheal injection of 10 μg. living B.C.G. and 100 mg. coal-mine dust. Large area of cellular proliferation with big dust aggregates. (H.E. × 175.)

FIG. 8.—365 days after intratracheal injection of 10 μg. living B.C.G. and 100 mg. coal-mine dust. Focal areas of dust collection and thickened alveolar septa. (H.E. × 42.)



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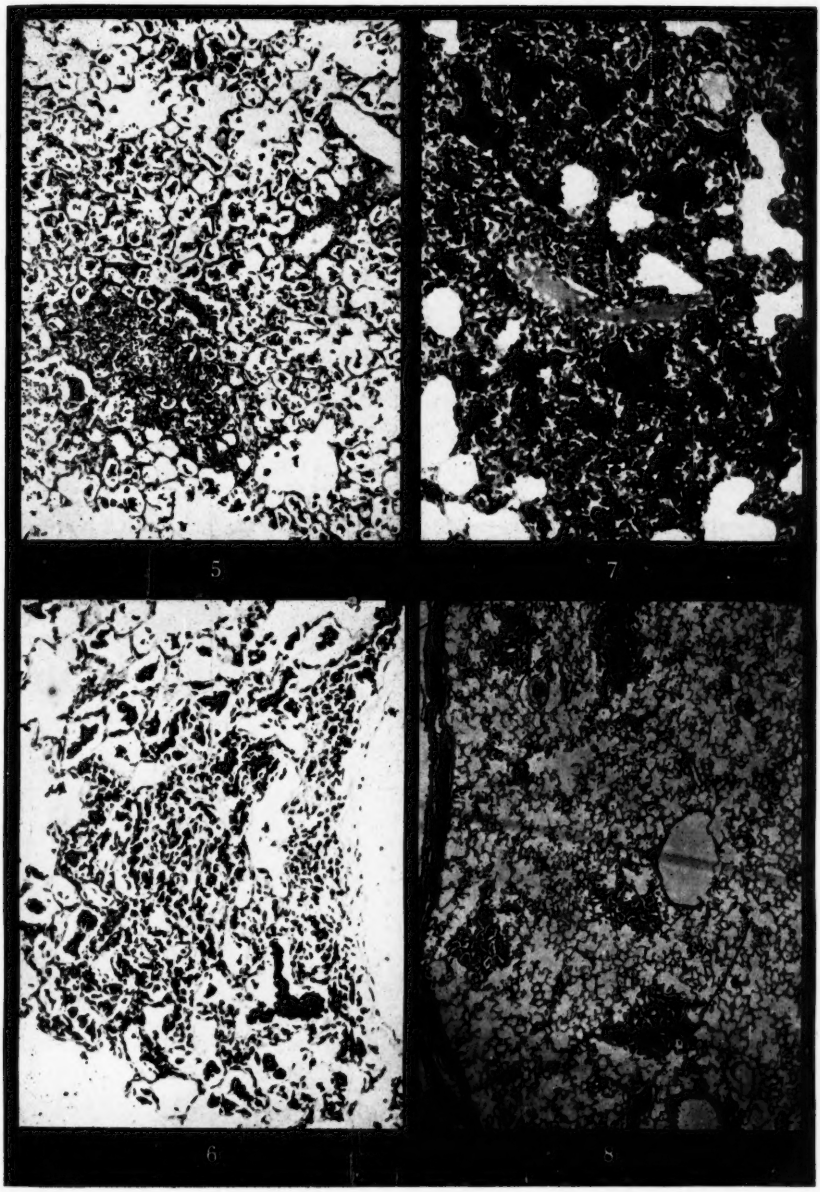

Zaidi, Harrison, King and Mitchison.

Immunisation with B.C.G.

A total of 36 guinea-pigs of 300–350 g. weight were injected subcutaneously with 1 μg. (wet weight) of B.C.G. organisms suspended in saline. After 4 weeks, 0·1 ml. of a 1/100 dilution of old tuberculin was injected intradermally on the flank; 48 hr. later all animals showed an erythematous and indurated zone at least 10 mm. in diameter at the site of injection.

Intratracheal injection.

The immunised guinea-pigs were divided into three groups, each containing 12 animals, and 3 days after tuberculin testing they were injected intratracheally by the method of Kettle and Hilton (1932) modified by Belt and King (1945). The materials injected were:

Group I: 100 mg. coal-mine dust in 2 ml. saline.
Group II: 10 μg. (wet wt.) B.C.G. in 2 ml. saline.
Group III: 10 μg. (wet wt.) B.C.G. and 100 mg. coal-mine dust in 2 ml. saline.

Overalls and face-masks were worn by the operators, and the nostrils and mouths of the animals were covered with lint to prevent exposure of the operators to regurgitated fluid.

Pathological techniques.

Guinea-pigs were killed at intervals of 1, 2, 3, 4, 5, 7, 9, 10 and 12 months after intratracheal injection, and full autopsy examinations were carried out. The lungs were fixed by intratracheal injection of about 15 ml. of 10 per cent formol-saline. After fixation, blocks for sectioning were taken along the coronal plane through the hilum to include hilar lymph nodes and maximal representative areas of lung tissue. The following staining techniques were employed: (1) haematoxylin and eosin, (2) silver impregnation for reticulin fibres (Gordon and Sweets, 1936), (3) Ziehl-Neelsen, (4) auramine for detection of acid-fast bacilli by fluorescence microscopy (Norman and Jelks, 1945).

TABLE—*Size-distribution and Analysis of Dusts used for Intratracheal Injection in Guinea-pigs.*

Anthracite.			Shale.		
Size in μ.	Per cent by number.	Per cent by mass.	Size in μ.	Per cent by number.	Per cent by mass.
0–1	89·4	6	0·5	1·4	0·02
1–2	7·3	13	1–2	18·7	2·3
2–3	2·2	18	2–3	73·6	74·1
3–5	1·4	48	3–4	6·0	20·6
5–7·5	0·1	15	4–5	0·2	1·6
			5–6	0·1	1·6

Composition:			
Coal	97·6 per cent.	Mica	51 per cent.
Ash	2·4 „ „	Quartz	31 „ „
		Kaolin	6 „ „
		Carbonates	7 „ „
		Ash	99·22 „ „

RESULTS.

Macroscopic Appearances.

At autopsy none of the animals showed lesions in any of their organs apart from the lungs. Those animals which had received dust, whether with or without tubercle bacilli, showed focal collections of black dust on the pleural and cut surfaces of the lungs. No other lesions were seen.

Microscopic Appearances.

Coal-mine dust alone (Group I).

In the guinea-pig killed at 30 days dust particles lay freely in the alveoli, with commencing aggregation (Fig. 1). Aggregation of dust particles and clearing of the intervening lung increased steadily. After 180 days the aggregates were compact (Fig. 2) and held together by a few fine reticulin fibres.

Tubercle bacilli alone (Group II).

A few scattered foci were seen in the lungs of the guinea-pig killed at 30 days. These foci (Fig. 3) showed thickening of the alveolar walls due to infiltration by macrophages, with a few polymorphs, eosinophils and an occasional giant cell. At 60 days the cellular reaction had increased. At 90 days and after the lesions had regressed, leaving essentially normal lungs (Fig. 4).

Coal-mine dust and tubercle bacilli (Group III).

At 30 days there was the same degree of aggregation of dust particles as in Group I. About half of the dust foci showed thickening of the alveolar walls, similar to that found in Group II, and appeared to be combined dust and bacterial lesions (Fig. 5). There was, however, no evidence that the dust had modified the tuberculous lesions. In the guinea-pig killed at 60 days, there was a similar aggregation of dust particles as was found in Group I, and a few foci (Fig. 6) showed a tuberculous reaction like that seen in the corresponding animal of Group II. The tuberculous reaction was always combined with dust aggregates, suggesting that dust and bacilli were transported by the same mechanism.

At 90 days the dust was well aggregated into foci. A minority of these foci were indistinguishable from those in the corresponding animals of Group I. However, the majority were combined foci and showed an infiltration of macrophages and lymphocytes into their alveolar walls. At 120 and 150 days most of the foci were combined ; the cellular infiltration was more intense, hiding the alveolar pattern, and a loose reticulin network was present (Fig. 7). At 210 days the foci were nearly all combined, and appeared to have regressed. They were smaller and most of the cellular reaction had disappeared, leaving thickened alveolar walls containing increased reticulin. Animals killed at 270, 300 and 365 days showed essentially similar lesions (Fig. 8).

In this group the presence of dust appeared to have prolonged the reaction to B.C.G. In the control animals of Group II, a reaction to B.C.G. was not seen after 60 days. In contrast, the reaction in the combined lesions appeared more severe, did not reach its maximum until 120–150 days, and then regressed, leaving slight fibrosis.

DISCUSSION.

Tubercle bacilli of the attenuated B.C.G. strain, when injected alone, produced regressive lesions which reached their peak at 60 days and then diminished until essentially normal lungs were found in animals killed at 90 days or after. Similar results were obtained by Vorwald, Dworski, Pratt and Delahant (1950). No reticulin was laid down within these lesions. Coal-mine dust alone also produced no fibrosis, which is in agreement with previous experience in this laboratory, even

though the dust contained 20 per cent of siliceous matter, mainly mica, quartz and kaolin. However, in animals injected with both dust and bacilli the majority of lesions were of a combined type in which the maximum cellular reaction occurred at 120–150 days and then regressed slightly. This prolongation of the reaction was accompanied by the deposition of loose reticulin fibres within the lesions.

Attygalle *et al.*, (1954) injected non-immunised rats and guinea-pigs with dead B.C.G. organisms alone or in combination with a coal-mine dust of similar composition to the one we have employed. In contrast to our findings the lesions produced in their experiment by the dead organisms alone were maximal in size at about 150–180 days and persisted for at least 500 days. Furthermore, the combined lesions in animals given bacilli and dust contained numerous collagen fibres at the peak period of fibrosis. These differences might be accounted for either because they injected a weight of bacilli approximately 150 times greater than we used or possibly because in immunised animals the mobilisation of macrophages which phagocytose and destroy the bacilli is accelerated (Lurie, 1936, 1939).

SUMMARY.

The combined action of coal-mine dust and living tubercle bacilli (B.C.G.), and of their individual effects, have been studied in the lungs of sensitised guinea-pigs.

Coal-mine dust produced no pulmonary fibrosis. B.C.G. alone caused proliferative lesions which reached a peak at 60 days, followed by resolution, with no evidence of disease after 150 days.

Coal-mine dust combined with B.C.G. produced proliferative and extensive lesions. The peak of disease was reached at 120–150 days and then regressed gradually over a period of one year. There was a loose reticulinosis of the lesions and no necrosis.

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EXPERIMENTAL INFECTIVE PNEUMOCONIOSIS. III: COAL-MINE DUST AND ISONIAZID-RESISTANT TUBERCLE BACILLI OF MODERATE VIRULENCE.

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ATTEMPTS to imitate human tuberculosis in the guinea-pig have been hindered by their high susceptibility to virulent strains of *Mycobacterium tuberculosis*. However, some isoniazid-resistant strains have been shown to have a reduced virulence for guinea-pigs, when injected by the intramuscular route, and to produce minimal lesions which tend to heal spontaneously, as do minimal lesions in the majority of human lungs (Barnett, Bushby and Mitchison, 1953*a*, *b*; Barry, Conalty and Gaffney, 1953; Middlebrook and Cohn, 1953; Peizer, Widelock and Klein, 1953; Steenken and Wolinsky, 1953; Mitchison, 1954; Karlson and Ikemi, 1954). We have therefore investigated the progress of disease produced in the lungs of guinea-pigs by intratracheal injection of an isoniazid-resistant strain with the hope of producing a disease closely resembling human pulmonary tuberculosis.

The relationship between tuberculosis and coal-mine dust has been studied in experimental animals to a limited extent. Cummins (1940), King, Harrison, Mohanty and Mitchison (1953) and Attygalle, Harrison, King and Mohanty (1954) have shown that tubercle bacilli combined with coal-mine dust cause larger lesions in the lungs of animals than do either coal-mine dust or tubercle bacilli alone. It was therefore considered desirable to study the influence of coal-mine dust on the lesions produced by isoniazid-resistant tubercle bacilli growing in the lungs of guinea-pigs. An attempt was also made to study the influence of coal-mine dust on the *in vitro* rate of growth of the isoniazid-resistant strain used in our animal experiments.

MATERIALS AND METHODS.

Tubercle bacilli.

Strain 3073 of *Myco. tuberculosis*, described by Barnett *et al.* (1953*a*, *b*) was used. This strain was isolated from the sputum of a patient with pulmonary tuberculosis, who had been treated at different times with *p*-aminosalicylic acid, streptomycin and, finally, isoniazid. It was resistant to all three drugs, growing on Löwenstein-Jensen slopes containing 10 μg./ml. isoniazid, but not on 50 μg./ml. Prior to our experiments, it had been sub-cultured on Löwenstein-Jensen slopes containing 1 μg./ml. isoniazid to prevent admixture with bacilli of lower resistance, but during the course of the experiments it was maintained by sub-culture on drug-free slopes. It had no catalase activity when tested by the technique of Middlebrook (1954). Barnett *et al.* (1953*b*) found that this strain was slightly more virulent for guinea-pigs than those with the highest degree of resistance to isoniazid that were tested. Suspensions of bacilli for intratracheal injections were prepared as described previously (Zaidi, Harrison, King and Mitchison, 1955).

Coal-mine dust.

The preparation of an artificial coal-mine dust for intratracheal injection has been described in the previous paper (Zaidi *et al.*, 1955).

Animal experiments.

Guinea-pigs of 300 g. average weight were injected intratracheally by the technique previously described. The animals were divided into five groups as follows :

Group I : 12 guinea-pigs each receiving 100 mg. coal-mine dust in 2 ml. saline. This group is the same as the control group previously described (Zaidi *et al.*, 1955).

Group II : 11 guinea-pigs each receiving 10 μg. (wet wt.) tubercle bacilli in 2 ml. saline.

Group III : 12 guinea-pigs each receiving 100 mg. coal-mine dust and 10 μg. (wet wt.) tubercle bacilli in 2 ml. saline.

Group IV : 12 guinea-pigs each receiving 5 μg. (wet wt.) tubercle bacilli in 2 ml. saline.

Group V : 12 guinea-pigs each receiving 100 mg. coal-mine dust and 5 μg. (wet wt.) tubercle bacilli in 2 ml. saline.

The animals in Groups I, II and III were injected at the same time, and the animals in Groups IV and V were injected together at a later date.

Pathological technique.

Procedures for autopsy and histological examination of the lungs were as described by Zaidi *et al.* (1955). A quantitative estimate of the extent of lung lesions was obtained by staining lung sections with haematoxylin and eosin, and then enlarging them six times on photographic paper. The whole lung area was cut out with scissors and weighed. Since the lesions were discrete, their images could be cut out and weighed. The percentage of the diseased area of the lungs was calculated as :

$$\frac{\text{weight of diseased areas of lungs}}{\text{weight of entire area of lungs}} \times 100.$$

Since the sections were cut in the same plane and position, these represented a similar sample of the disease process in each guinea-pig.

In vitro *experiments.*

Duplicate sets of screw-cap bottles containing Tween-albumin medium (Medical Research Council, 1953), with and without the addition of 100 mg. sterile coal-mine dust, were inoculated with an amount of a 10-day culture of tubercle bacilli growing in Tween-albumin medium, sufficient to make a final dilution of 1/1500. The bottles were then incubated at 37°, and standard 3 mm. loopfuls (0·005 ml.) of the cultures were spread on Löwenstein-Jensen slopes after 0, 2, 4, 6, 8, 11 and 13 days. After 14 days, loopfuls from 1/10, 1/100 and 1/1,000 dilutions of the cultures were spread on further slopes. The Löwenstein-Jensen slopes were incubated 28 days and the amount of growth on them was assessed. A similar procedure was carried out using 50 per cent guinea-pig serum in distilled water in place of Tween-albumin medium, partly to simulate the *in vivo* conditions more nearly, and partly to provide an excess of factors necessary for the growth of some isoniazid-resistant strains (Fisher, 1954).

RESULTS.

Mortality.

The survival and mode of death of the guinea-pigs are shown in the Table.

Coal-mine dust alone (Group I).

Animals were killed at approximately monthly intervals up to 365 days after intratracheal injection. There was no mortality referable to dust.

Tubercle bacilli alone (*Groups II and IV*).

Only 3 of the 11 animals receiving 10 μg. bacilli died of tuberculosis. The remaining 8 were killed at intervals up to 365 days. Similarly, 3 of those that received 5 μg. bacilli died of tuberculosis. A fourth died at 104 days from coincidental disease. The remaining 8 were killed at intervals up to 150 days.

Coal-mine dust and tubercle bacilli (*Groups III and V*).

Of the 12 animals receiving 100 mg. dust and 10 μg. bacilli, 11 died between 14 and 42 days. The twelfth guinea-pig was killed at 60 days, when it was found that part of the dose had been injected outside the trachea. Similarly, 10 out of 12 animals that received 100 mg. dust and 5 μg. bacilli died between the 22nd and 69th days. One animal was killed at 30 days to obtain satisfactory histology. The remaining animal was killed on the 90th day, and was again found to have been misinjected. Thus the combination of dust and bacilli was uniformly fatal when the technique of injection was satisfactory.

Macroscopic Appearances.

Tuberculous lesions were confined to the lungs.

Coal-mine dust alone (*Group I*).

The dust lay in focal collections, visible on the pleural and cut surfaces of the lungs, and evenly distributed throughout the lobes. There was no evidence of fibrosis.

Tubercle bacilli alone (*Groups II and IV*).

In animals given either 10 or 5 μg. of bacilli, and dying or killed before 90 days, scattered tubercles of 1–2 mm. diameter were seen on the pleural and cut surfaces of the lungs, and sometimes areas of consolidation occurred in the lower lobes. No tubercles were seen in the guinea-pigs killed at 90 days or later. However, foci of fibrosis, often calcified, could be detected from 120 days onwards.

Coal-mine dust and tubercle bacilli (*Groups III and V*).

In animals dying or killed up to 60 days after injection the lungs were largely consolidated and black in colour. In both groups from 60 days onwards cavities of variable size, up to 10 mm. diameter, were present.

Microscopic Appearances.

Coal-mine dust alone (*Group I*).

The results in this group have been previously described and illustrated (Zaidi *et al.*, 1955). After 30 days the dust was well aggregated into foci, which, after 180 days, contained a few fine reticulin fibres.

Tubercle bacilli alone (*Groups II and IV*).

Lesions before 60 days were largely focal and consisted of consolidation which was partly exudative and partly interstitial, composed mainly of macrophages and polymorphs without any epithelioid cells or giant cells (Fig. 1, 2). There was

reticulin thickening of the alveolar walls. From about 60 days onwards the exudative reaction largely disappeared, leaving foci of fibrosis, which were smaller and less frequent in the animals killed latest (Fig. 5, 6). This tendency is illustrated in Table I, which shows the quantitative estimates of the extent of the lung lesions. Small areas of central necrosis were frequent in the two animals killed at 120 days in Group II and in the animals killed at 30, 67, 90, 104 and 150 days in Group IV.

In animals killed or dying before 60 days, large numbers of acid-fast bacilli were seen in the lesions. At 60 days, the numbers were less and after this none were found in any of the lungs.

Coal-mine dust and tubercle bacilli (Groups III and V).

One of the striking features of the animals in these groups was the high proportion of the lung tissue involved in lesions. This is illustrated in the paired Figures 1–8. In the Table it can be seen that with 10 μg. of bacilli and dust an average of 68 per cent of the lungs was consolidated, and with 5 μg. of bacilli and dust an average of 82 per cent. The corresponding figures for animals infected with bacilli alone and surviving for corresponding periods were 37 per cent and 32 per cent respectively.

Another feature of interest was the striking similarity of the lung lesions in animals dying at about the same period, so that it was possible to obtain a clear picture of the progress of the disease.

In animals dying 14–20 days after injection, lesions were rounded and only occasionally confluent. They consisted of an exudate and an interstitial infiltration composed of macrophages and polymorphs. There was a slight increase in reticulin but no necrosis.

After 20 days the lesions were confluent (Fig. 3, 4) with areas of necrosis and an increasing number of reticulin fibres. In animals surviving for 60 days or more there were cavities surrounded by a cellular fibrosis still containing inflammatory cells (Fig. 7 and 8).

Acid-fast bacilli were present in large numbers in the lesions of all animals in these two sub-groups, including those dying at 60 days or after.

EXPLANATION OF PLATES.

FIG. 1.—Guinea-pig lung, killed 30 days after intratracheal injection of 5 μg. strain 3073. Few scattered tubercles. (H.E. × 3·8.)

FIG. 2.—48 days after intratracheal injection of 10 μg. strain b3073. Fibrocaseous cavities. (H.E. × 3·8.)

FIG. 3.—30 days after intratracheal injection of 5 μg. strain 3073 plus 100 mg. coal-mine dust. Extensive lesions produced by the combined action of strain 3073 and coal-mine dust. (H.E. × 3·8.)

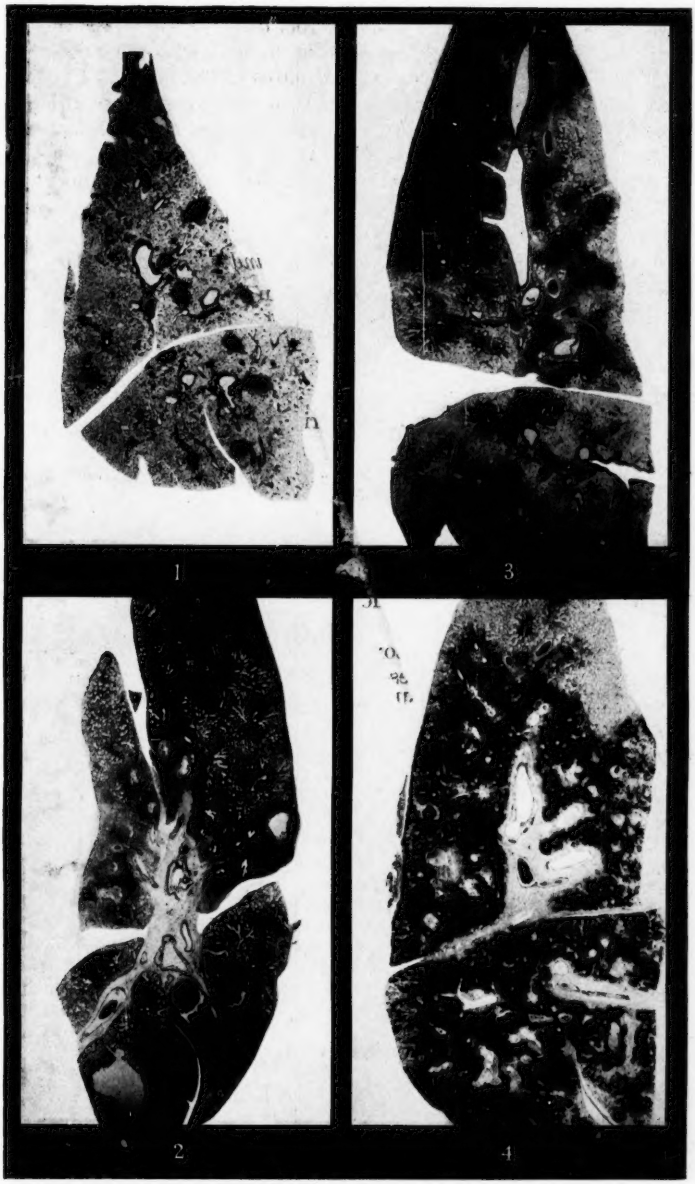
FIG. 4.—42 days after intratracheal injection of 10 μg. strain 3073 plus 100 mg. coal-mine dust. The whole lung area except a small portion is involved. (H.E. × 3·8.)

FIG. 5.—Guinea-pig lung, killed 60 days after intratracheal injection of 10 μg. strain 3073 alone. Large areas of necrosis. (H.E. × 3·8.)

FIG. 6.—67 days after intratracheal injection of 5 μg. strain 3073. An area of necrosis and few scattered areas of consolidation. (H.E. × 3·8.)

FIG. 7.—60 days after intratracheal injection of 10 μg. strain 3073 plus 100 mg. of coal-mine dust. Large cavities lined by thick reticulin fibres and few collagen fibres.

FIG. 8.—69 days after intratracheal injection of 5 μg. strain 3073 + 100 mg. coal-mine dust. Cavities occupying the whole parenchyma of the lungs. The cavity walls are composed of collagen and reticulin fibres. (H.E. × 3·8.)



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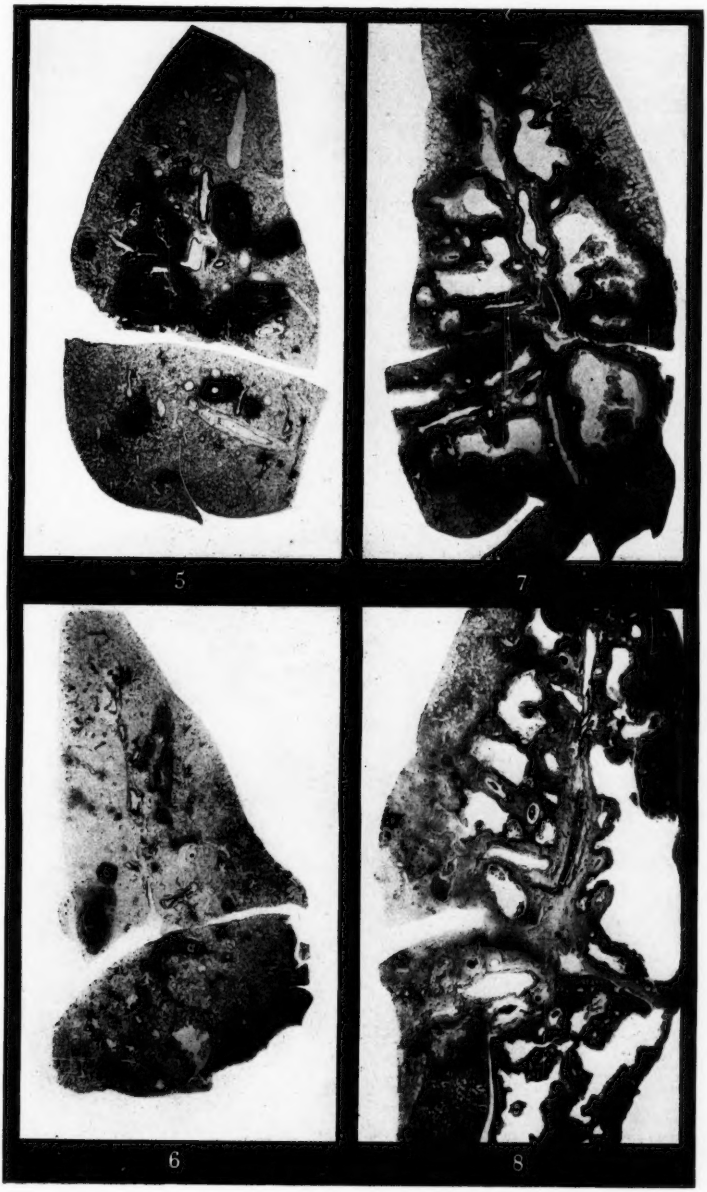

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TABLE.—*Per Cent Diseased Area on Lung Section of Guinea-pigs Injected with Coal-mine Dust, Tubercle Bacilli or Both.*

100 mg. Coal-mine dust.			10 μg. Strain 3073.			100 mg. Coal-mine dust + 10 μg. Strain 3073.			5 μg. Strain 3073			100 mg. Coal-mine dust + 5 μg. Strain 3073.		
Days.	Mode of death.	Average % diseased area.	Days.	Mode of death.	Average % diseased area.	Days.	Mode of death.	Average % diseased area.	Days.	Mode of death.	Average % diseased area.	Days.	Mode of death.	Average % diseased area.
30	K	0	23	D	31	14	D	51	19	D	54	22	D	73
60	K	0	48	D	58	15	D	58	30	K	15	26	D	77
90	K	0	60	K	21	15	D	76	41	D	80	29	D	—
120	K	0	66	D	45	23	D	69	42	D	—	30	K	81
150	K	0	90	K	18	23	D	68	67	K	23	37	D	77
180	K	0	120	K	31	25	D	65	67	K	27	38	D	70
210	K	0	120	K	45	26	D	58	90	K	11	45	D	88
240	K	0	150	K	9	26	D	70	90	K	19	58	D	—
270	K	0	150	K	32	27	D	80	104	D†	21	60	D	—
280	D*	0	300	K	3	27	D	75	150	K	11	67	D	94
300	K	0	365	K	3	42	D	79	150	K	10	69	D	93
365	K	0				60	K	78	150	K	2	90	K	29

D = died ; K = killed.

* = died with liver abscess.

† = non-tuberculous death.

In vitro *experiments.*

The rate of growth of the bacilli in Tween-albumin medium with dust was rather slower than without dust, and there was a fall in the number of viable bacilli between the 11th and 14th days. It is possible that both the slow growth and poor viability in the presence of dust were due to the adsorption by dust particles of growth factors which were only present in minimal amounts in this medium (Fisher, 1954).

In the 50 per cent guinea-pig serum medium, which presumably contained an excess of growth factors, the rate of growth and the final count after 14 days' incubation were similar, irrespective of the presence of dust.

DISCUSSION.

Gardner (1922) produced experimental pulmonary tuberculosis in guinea-pigs with the attenuated R1 strain, and from histopathological studies of the lungs he showed that the lesions healed by resolution. Steenken, Wolinsky, Pratt and Smith (1952) produced chronic discrete lesions in the lungs of guinea-pigs following lengthy treatment with streptomycin. The lesions produced were generalised in the lung, liver and spleen, and often associated with cyst-like spaces filled with purulent material. These lesions did not show any giant cell formation or epithelioid cells.

In our experiments with strain 3073 alone the lesions in the lungs of guinea-pigs at 60 days resembled healing human fibrocaseous lesions in that they tended to heal by calcification and fibrosis, diminished in size, but did not resolve up to a period of one year. The equilibrium in host-parasite relationship found between strain 3073 and guinea-pig lung tissue seems to resemble that found in human fibrocaseous disease.

Coal-mine dust alone did not cause any fibrosis up to 365 days. During this period the dust particles collected in nodules held together by very fine reticulin fibres.

Experimental studies have shown some relationship between dusts and tuberculous infection. Kettle (1924, 1934), Gardner (1929), Dowd (1935), Vorwald and Delahant (1938), Vorwald, Dworski, Pratt and Delahant (1950) have shown that tubercle bacilli, either dead or attenuated, produce proliferating lesions in the presence of silica. Gardner (1929), Policard (1939), Sen (1939), Vorwald *et al.* (1954), on the other hand, have shown reactivation of latent tuberculous infection leading to progressive and fatal tuberculosis as a complication of the silicotic process in the lungs of animals. With " inert " dusts, those which are much less fibrogenic than silica, there is also experimental evidence that tubercle bacilli flourished in dust lesions and produced extensive tuberculosis. Gardner (1923) studied the effects of carborundum dust inhalations on tuberculous animals and found that the self-limiting tuberculosis produced by the R1 strain, which heals by resolution, was transformed into a progressive tuberculous bronchopneumonia. Kettle (1932) studied the interstitial reaction by injecting suspensions of silica dust and kaolin subcutaneously, followed by living tubercle bacilli intravenously, and found that the bacilli proliferated in the dust lesions. Cummins (1940), and Attygalle *et al.* (1954) produced much larger and more cellular lesions with anthracite dust and dead tubercle bacilli than with either alone. Cummins (1940)

suggested that these amplified lesions were " due to the action of dust and bacilli acting together ".

In the present series of experiments, 100 mg. coal-mine dust + 5 or 10 μg. of strain 3073 were injected intratracheally. These combinations killed all the guinea-pigs in 69 days and 42 days respectively. The histological picture, during the first two months, showed extensive tuberculous lesions consisting of broncho-pneumonic patches and caseating areas with numerous dust particles. Cavitation, thickening of reticulin, collagen fibres and some well fibrosed areas were seen later. In both the series almost the whole lung area was involved and the extent of the lesions was much greater than with strain 3073 alone. The fibrosis was mature, showing thick reticulin and collagen fibres, whereas in animals infected with strain 3073 alone the reticulin was thin and collagen fibres were scanty.

The *in vitro* experiments with coal-mine dust and strain 3073 show that the dust, under the conditions used, had no stimulating action on the growth of strain 3073. Price (1932) and Gardner (1938) showed enhancement of the growth of tubercle bacilli in the presence of silica. Kettle (1932) suggested that the proliferation of tubercle bacilli is due to the solubility of silica, the silicic acid combining with the nutrient substances to form a rich medium for the growth of tubercle bacilli. However, this theory cannot be accepted as an explanation here, since coal-mine dust contains very little crystalline silica, and the solubility of this is greatly depressed by the action of mica and kaolin. Hence there can be no appreciable formation of silicic acid to provide a medium for the growth of tubercle bacilli. A satisfactory explanation of how dust particles and tubercle bacilli reinforce each other's biological effects, to produce the severe and extensive lesions found in these experiments, cannot yet be given.

SUMMARY.

The combined action of coal-mine dust and tuberculous infection produced by the catalase-negative isoniazid-, streptomycin- and P.A.S.-resistant strain 3073 of *Myco. tuberculosis* of moderate virulence, and the individual effects of strain 3073 and coal-mine dust, have been studied by the intratracheal injection of these substances in guinea-pigs.

Strain 3073 alone produced fibrocaseous lesions which regressed, leaving small calcified nodules as the only evidence of previous infection. Coal-mine dust alone produced no fibrosis.

The combined action of bacilli and dust produced extensive tuberculous cavities involving large areas of lung tissue, and killed the guinea-pigs.

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EXPERIMENTAL INFECTIVE PNEUMOCONIOSIS. IV: MASSIVE PULMONARY FIBROSIS PRODUCED BY COAL-MINE DUST AND ISONIAZID-RESISTANT TUBERCLE BACILLI OF LOW VIRULENCE.

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CUMMINS and Sladden (1930) described asymmetrical and irregular areas of consolidation demonstrable by X-ray in the lungs of coal-miners. These opacities were blurred and irregular in outline and lacked the density seen in silicotic nodules at autopsies of coal-miners. Gough (1947) found massive fibrosis, often unilateral, sometimes bilateral and most frequently in the upper and posterior part of the lung. These masses of fibrosis, as Gough pointed out, are like lumps of coal, rubbery in consistency and several inches in diameter. Sometimes ragged cavities are present in the centre of these lesions, and contain black inky fluid and cholesterol crystals. Chemical analysis of these lungs showed the percentages of silica and coal to be only slightly above those of simple pneumoconiosis (King and Nagelschmidt, 1945; 1955). Belt and Ferris (1942) suggested a sporadic factor X which caused massive fibrosis of the lungs of coal-miners and pointed out that there was some reason for thinking of factor X as biological, possibly tubercle bacilli. They thought that " tuberculous infection might be playing an unsuspected role, acting under cover of dust, which hitherto remained unidentified ". Gooding (1946) reported 26 per cent positive cases of tuberculosis among 230 autopsies of miners with massive fibrosis and Rogers (1946) found 35 per cent gross tuberculous lesions in the lungs of South Wales miners. Gough (1949) suggested that the massive fibrosis was due to the combined action of infection and dust. However, in only 40 per cent of cases of massive fibrosis could tubercle bacilli be demonstrated in the lesions post mortem or by culture or animal inoculation (James, 1954).

Experimental studies showing that the combined action of dust and tubercle bacilli causes fibrotic lesions are described, and references given, in the two preceding papers.

The reasons for employing an isoniazid-resistant strain of tubercle bacilli to simulate human pulmonary tuberculosis have been given. In the present experiments a strain of still lower virulence for guinea-pigs than strain 3073 has been used, since the aim was to produce a milder infective stimulus over a long period, which would not kill the animals. For this purpose a strain was used which had been made resistant to isoniazid *in vitro*. The combined action of infection with this organism and coal-mine dust produced massive fibrosis in the lungs of guinea-pigs.

MATERIALS AND METHODS.

Tubercle bacilli.

An isoniazid-resistant variant of the virulent human strain, H37Rv, described by Barnett, Bushby and Mitchison (1953*a*, *b*) as B was used. Strain B grew on Löwenstein-Jensen slopes containing 200 μg./ml. isoniazid, but not on 500 μg./ml., and was catalase-negative when tested by the Middlebrook (1954) technique. This strain was found by Barnett *et al.* (1953*b*) to be less virulent in guinea-pigs than the strain 3073 used in the previous experiments. This reduced virulence was confirmed in a preliminary experiment, in which groups of 5 guinea-pigs were injected intratracheally with 10, 1, 0·1 or 0·01 μg. (wet wt.) of strain B together with 100 mg. coal-mine dust. Of the 5 animals each injected with 10 μg. strain B and 100 mg. dust only one had died after 42 days, whereas in the experiment where 10 μg. of strain 3073 and 100 mg. dust had been injected, 11 out of 12 animals had died after 42 days. The extent of the lesions produced in the lungs was proportional to the dose of bacilli injected. A dose of 1 μg. of strain B was chosen for the main experiment since it produced discrete lesions and might allow prolonged survival of the animal.

The method for preparing a suspension of bacilli for intratracheal injection has been described previously.

Coal-mine dust.

The composition of the dust employed and its preparation for intratracheal injection are described in the preceding papers.

Animal experiments.

Guinea-pigs of average weight 300 g. were injected intratracheally with the following materials :

Group I : 12 guinea-pigs each receiving 100 mg. coal-mine dust. This group is the same as the control group previously described (Zaidi, Harrison, King and Mitchison 1955*a*) and was injected at an earlier period than Groups II and III.

Group II : 26 guinea-pigs each receiving 1 μg. (wet wt.) of tubercle bacilli in 1·5 ml. saline.

Group III : 26 guinea-pigs each receiving 100 mg. coal-mine dust and 1 μg. (wet wt.) of tubercle bacilli in 1·5 ml. saline.

The methods for performing intratracheal injection, autopsies and histological examinations have been described. In addition, an estimate of the number of viable tubercle bacilli in the lungs of the guinea-pigs, at intervals up to 150 days, was obtained. At autopsy, the cardiac lobe of the lungs from each animal was taken out with aseptic precautions, and was ground in a sterile Potter-Elvejhem grinding tube with water to make up the volume to 1 ml. Serial tenfold dilutions of the suspension were made in distilled water, and a standard 3 mm. loopful was transferred from the dilutions to two Löwenstein-Jensen slopes. The number of colonies on the slopes was counted after incubation for 28 days.

Animals were killed at monthly intervals until 300 days after injection, In Group II (bacilli alone) one guinea-pig died of intercurrent infection 26 days after injection. In Group III (bacilli + dust) one animal died 2 days after injection, and 5 more died after 80 days, during a short period of very cold weather. The pulmonary lesions of the latter animals did not appear sufficiently extensive to account for their death, nor was any other cause found.

Number of tubercle bacilli in the lungs.

The results of the viable counts on the lungs of the guinea-pigs are shown in Fig. 1. On the day after injection similar counts were obtained from animals in Groups II and III. In Group II (bacilli alone) no growth was obtained from the lungs of animals killed at 30 days or subsequently, whereas the counts from the animals in Group III (bacilli + dust) reached a peak at 30 days and gradually declined until, at 150 days, no organisms were recovered.

Virulence and resistance of recovered bacilli.

Tubercle bacilli cultivated from the lungs of a guinea-pig killed 60 days after injection of bacilli + dust were examined to see whether they had retained their original degree of viru-

lence and isoniazid resistance. Doses of 0·1 mg. (wet wt.) of this organism and of a subculture maintained on Löwenstein-Jensen medium, of the original culture used for infecting the animals were each injected intramuscularly into 3 guinea-pigs. They were killed after 56 days and were all found to have small abscesses at the site of injection and minimal caseation in the draining lymph nodes, but no other lesions. Thus there had been no increase in virulence as a result of passage through the guinea-pig in the presence of dust. The recovered culture had also retained its original degree of resistance to isoniazid, as measured by the Medical Research Council (1953) technique.

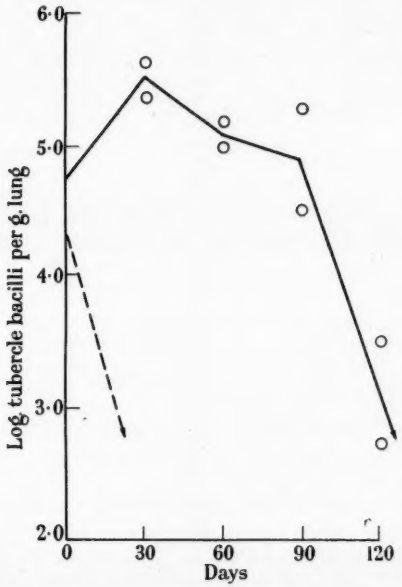


FIG. 1.—Bacillary counts in culture from lungs of guinea-pigs inoculated intratracheally with 1 μg. *Myco. tuberculosis* strain B, alone (- - - -) and together with 100 mg. coal-mine dust (———). The slope of the former was chosen to indicate that on day 30 cultures were sterile (the point on the ordinate being the minimum number of bacilli detectable by the method used).

RESULTS.

Macroscopic Appearances.

Coal-mine dust alone (Group I).

In animals given coal-mine dust alone the dust lay in focal collections, which were evenly distributed throughout the lobes. There was no evidence of fibrosis.

Tubercle bacilli alone (Group II).

Animals injected with 1 μg. of bacilli and killed at 30 and at 60 days showed a few greyish lesions of less than 1 mm. diameter. After 60 days no macroscopic lesions were visible.

Coal-mine dust and tubercle bacilli (Group III).

In the lungs of animals killed at 30 and 60 days there were coalescent dark grey areas of consolidation in which could be seen paler foci up to 2 mm. in diameter. About a third to a half of the lung tissue was consolidated. With the progress of time the lesions did not alter in size, but they became tougher in consistency.

Microscopic Appearances.

Coal-mine dust alone (Group I).

The results in this group have been previously described and illustrated (Zaidi *et al.*, 1955*a*). After 30 days the dust was well aggregated into foci which, after 180 days, contained a few fine reticulin fibres.

Tubercle bacilli alone (Group II).

In animals killed or dying at 1, 26 or 30 days after injection, there were scanty areas of pulmonary reaction. These consisted of an exudate principally of polymorphs at 1 day and of polymorphs and macrophages at 26 and 30 days. At 26 and 30 days there was also thickening of alveolar walls caused by proliferation of tissue histiocytes. At 60 days the lesions were smaller; the exudate had resolved, leaving only thickened alveolar walls. At 90 days and after no lesions were detected apart from occasional small calcified nodules (see Fig. 2–4).

Coal-mine dust and tubercle bacilli (Group III).

In the dust-laden parts of the lungs of the guinea-pig killed 24 hr. after injection the alveoli contained numerous dust-filled macrophages and the alveolar walls were thickened by swollen tissue histiocytes.

In the two animals killed at 30 days large areas of lung were consolidated (Fig. 5). This was caused partly by an exudate of macrophages and partly by a proliferation of tissue histiocytes within the alveolar walls. In the latter situation there was an increase of reticulin fibres, and there was also some growth of fibres in the consolidated alveolar spaces, so that in the more affected areas it was difficult to recognise the original lung pattern. By this stage the cell reaction was almost wholly of the macrophage-histiocyte group with only a few polymorphs.

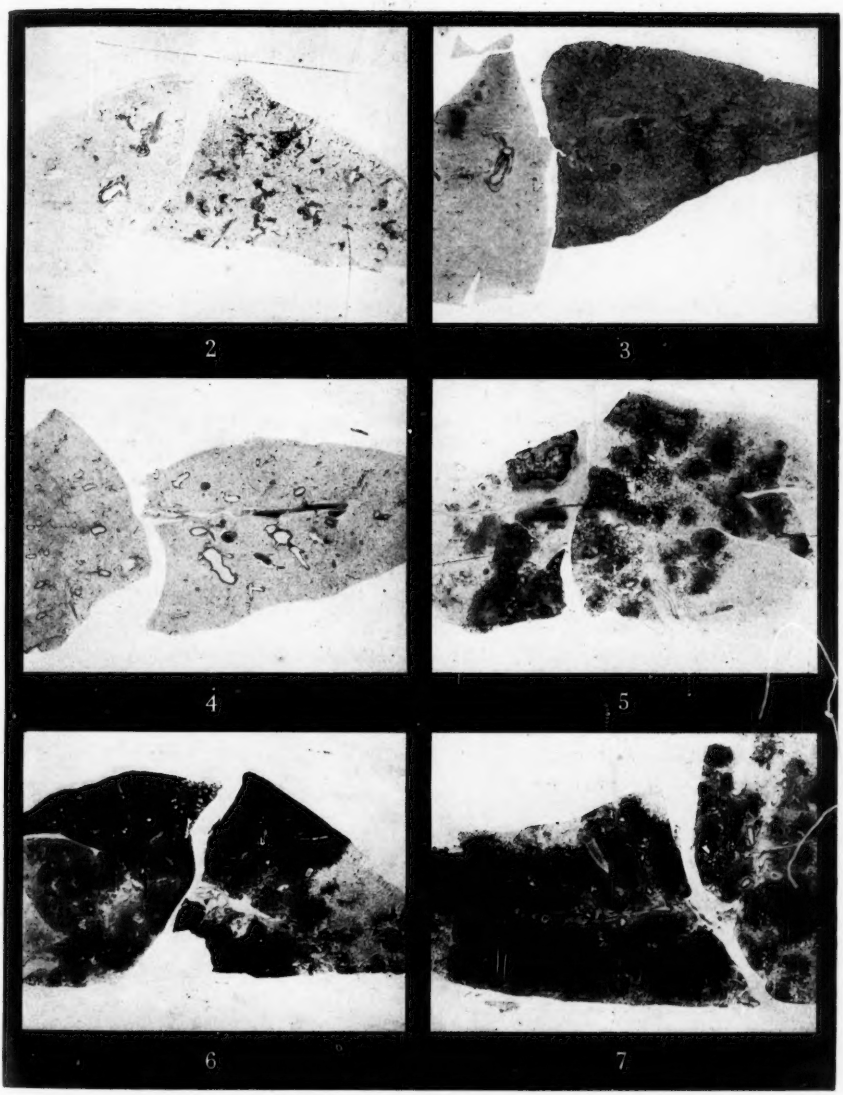
EXPLANATION OF PLATES.

FIG. 2–4. Guinea-pig lungs after the intratracheal injection of 1 μg. strain B *Myco. tuberculosis*. Haematoxylin and eosin. × 5. (2) 30 days: small circumscribed areas are seen scattered about the lung. (3) 60 days: very few lesions seen at this stage. Almost the whole lung parenchyma is normal. (4) 90 days: normal lung structure.

FIG. 5–7. Intratracheal injection of 1 μg. strain B *Myco. tuberculosis* + 100 mg. coal-mine dust. Haematoxylin and eosin. × 5. (5) 30 days: combined lesions constitute more than half the lung tissue. (6) 60 days: large areas of involvement of the lung tissue. (7) 90 days: the whole lung shows widespread lesions.

FIG. 8–10. Guinea-pig lungs after the intratracheal injection of 1 μg. strain B *Myco. tuberculosis* + 100 mg. coal-mine dust. Haematoxylin and eosin. × 5. (8) 120 days: widespread lesions. (9) 180 days: most of the lesions have cavities. (10) 300 days: the lesions occupy the whole of one lobe, and part of the other, and show no tendency to resolution.

FIG. 11–13.—Intratracheal injection of 1 μg strain B *Myco. tuberculosis* + 100 mg. coal-mine dust. (11) 120 days: higher magnification of Fig. 8. Loosely woven collagen and reticulin fibres and some adenomatosis. Silver impregnation. × 50. (12) 180 days: higher magnification of Fig. 9. The whole of large cavity lined with thick reticulin and collagen fibres. Silver impregnation. × 40. (13) 150 days: large number of acini lined by columnar epithelium (adenomatosis). Haematoxylin and eosin. × 200.



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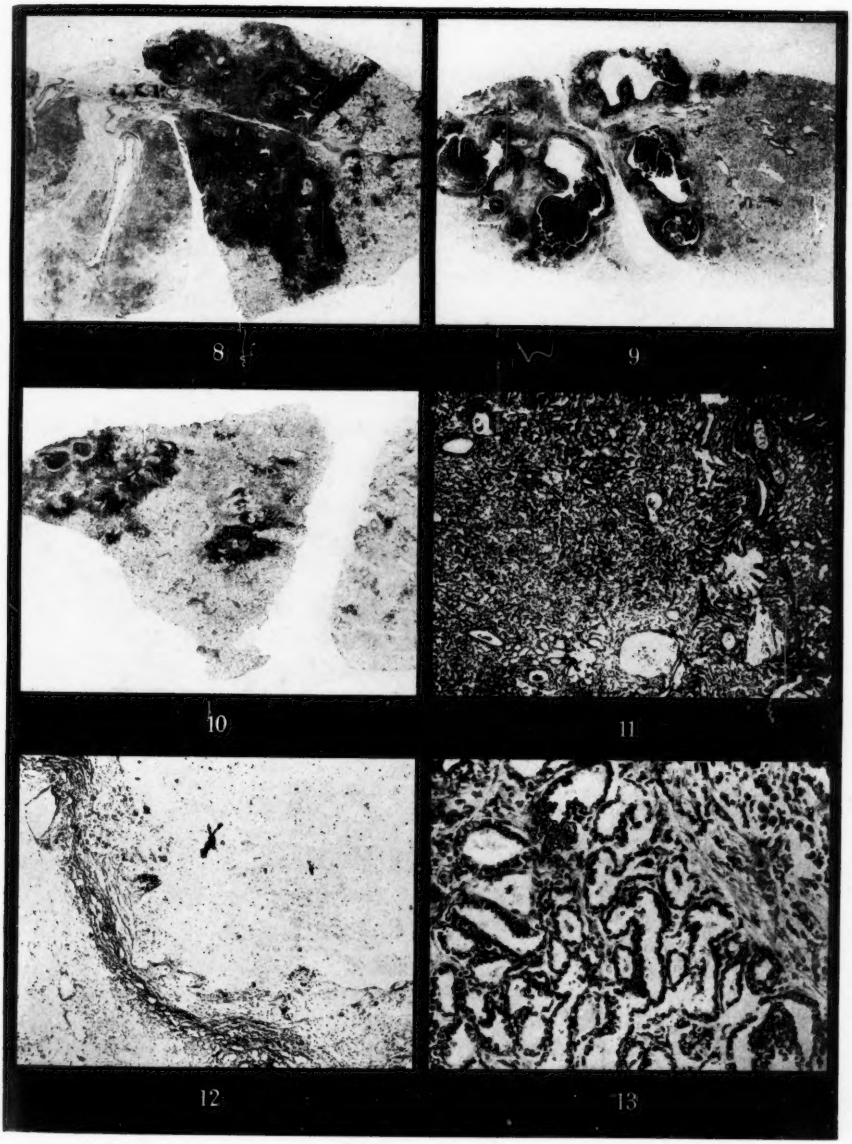

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In the centres of most of the areas of consolidation there were foci showing pyknosis of the nuclei, but rarely full necrosis. Most of the consolidated areas contained dust, but there were some foci without dust, and there were others containing phagocytosed dust without any tuberculous reaction.

Two animals killed at 60 days showed lesions essentially similar to those seen at 30 days (Fig. 6), but reticulin formation was greater, rendering the lung pattern quite invisible. Central necrosis was complete in some foci and absent in others.

In the five animals dying on the 80th and 81st days after inoculation the lesions were substantially similar to those seen at 60 days. They were on the whole rather more extensive, and in some, but not all, the reticulinosis was a little denser. In two animals foci of necrosis were breaking down.

In the animals killed at 90 days (Fig. 7) the lesions showed no significant change from those seen at 80 days. In two animals killed at 120 days (Fig. 8 and 11) and two more killed at 150 days the lesions were again similar except that foci of necrosis were more sharply defined and were calcified. In those killed after this and up to the end of the experiment at 300 days there was little change in the lesions. Some animals showed central necrosis with cavitation (Fig. 9 and 12) and, as a general rule, reticulin tended to be replaced by collagen. Some killed late in the experiment had rather smaller lesions, suggesting a possible shrinkage (Fig. 10).

An unexpected finding in this group was the occurrence of what we have called adenomatosis. This took the form of an overgrowth of bronchial epithelium. A minor degree of this was a local overgrowth forming small clusters of acini outside the bronchus : when it was more extensive the bronchial epithelium grew out into the lung tissue lining alveolar spaces quite widely.

The earliest and mildest example was seen at 60 days, and some degree of adenomatosis was found at every stage after this up to 300 days. The most extensive change was seen at 120 and 150 days (Fig. 13) and, after this, appeared to become slighter.

DISCUSSION.

In these experiments the combined action of dust and isoniazid-resistant tubercle bacilli produced massive fibrosis in the lungs of guinea-pigs, whereas tubercle bacilli alone or coal-mine dust alone did not produce any fibrosis. The lesion produced by the combined action of coal-mine dust and tubercle bacilli was composed of collagen fibres and thick and compact reticulin, and between these fibres coal-mine dust was deposited. In the later stages the centre of the lesions showed necrosis which often developed into cavities, lined in some cases by collagen fibres. There was no histological evidence of tuberculosis in these lesions, except that in guinea-pigs killed during the first five months tubercle bacilli could be demonstrated microscopically. In this respect the lesions resembled the massive fibrosis found in the lungs of coal-miners. The asymmetrical and irregular lesions produced in the lungs of the guinea-pigs by the combined action of coal and tubercle bacilli persisted up to 300 days. This failure to show any tendency to resolution is also seen in the fibrosis of the coal-miner's lung. In two small points, however, the massive fibrosis in the lungs of guinea-pigs differed from that found in the lungs of coal-miners : there was marked adenomatosis and calcification of the small cavities, possibly a species difference.

Although it was difficult to demonstrate tubercle bacilli in the lesions histologically, viable counts could be made and were highest at 30 days. The number of

tubercle bacilli then gradually fell and at 150 days and later none could be grown. In contrast the lungs of the guinea-pigs injected with strain B alone showed tubercle bacilli on the day following the injection, but no growth was obtained at any of the succeeding killings up to 300 days. In the presence of coal-mine dust tubercle bacilli multiplied in the lungs, but did not multiply in its absence. Bacilli recovered from the lesions showed no increase in virulence or change in sensitivity to isoniazid. Massive fibrosis, therefore, was not brought about by an increase in the virulence of the bacilli, and when fibrous tissue had formed the bacilli died out.

Cohn, Kovitz, Oda and Middlebrook (1954) have shown that catalase or pyruvate promotes the growth of catalase-negative isoniazid-resistant strains of tubercle bacilli. They suggest that such strains may lack the capacity to produce progressive disease because " in the sufficiently inflamed focus, low oxygen tension favours immediate reduction of any pyruvic acid to lactic acid, which has no growth-supportive activity for catalase–negative tubercle bacilli ". Furthermore, they believe that in open pulmonary cavities the presence of well-aerated, autolysing tissue provides a sufficient source of catalase for continued multiplication. In our experiments the number of bacilli in the lungs of animals infected with dust and bacilli increased during the phase of increasing cellular reaction and decreased when cavitation began to appear. These observations suggest that the lack of catalase activity of isoniazid-resistant strains may not be a sufficient explanation for their low virulence.

James (1954) found progressive massive fibrosis occurring in young men with short exposures to dust. The autopsy material from 11 such coal-miners in their 20's showed evidence of tuberculosis. On the other hand there was difficulty in demonstrating tubercle bacilli in older men ; and Cochrane, Cox and Jarman (1952) showed that in only 1 per cent of such cases during life did the sputum contain tubercle bacilli. Similarly, we could not demonstrate tubercle bacilli by culture of the lung or microscopically after 150 days. An intercurrent tuberculous infection superimposed on simple pneumoconiosis may cause massive fibrosis from which, in its early stages, tubercle bacilli can be isolated. The chances of demonstrating tubercle bacilli in such lungs would be greater than in the lungs of aged miners, where the lesions are old and in which the bacilli may have died out.

The work of Price (1932) on the enhancement of growth of tubercle bacilli by silica could not be substantiated by Vorwald, Dworski, Pratt and Delahant (1954). They suggested that silica did not cause rapid growth of bacilli by direct action upon them, but that when embedded in tissues it favours the growth of tubercle bacilli which become localised in these tissues (Vorwald and Delahant, 1938 ; Gardner, 1937). Furthermore, in a previous experiment (Zaidi *et al.*, 1955*b*) the presence of coal-mine dust did not stimulate the growth of isoniazid-resistant tubercle bacilli.

The ability of macrophages to destroy or inhibit the growth of bacilli may be affected when dust is injected with bacilli. The bacilli, apparently capable of further proliferation in presence of the dust, may provide a continued stimulus for the accumulation of larger numbers of macrophages and eventually the laying down of fibrous tissue.

SUMMARY.

The effects of intratracheal injection of coal-mine dust, a catalase-negative isoniazid-resistant strain of tubercle bacilli of low virulence, and a combination of

dust and bacilli were studied in the lungs of guinea-pigs. The bacilli alone produced lesions which tended to disappear after 90 days and the only evidence of tuberculosis at 300 days was a few calcified nodules in some of the lungs. Coal-mine dust produced no pulmonary fibrosis.

The combined action of coal-mine dust and bacilli produced massive pulmonary fibrosis. In its early stages the fibrosis was composed mainly of thick reticulin fibres and collagen but over a longer period more collagen was laid down.

Serial cultures of the lungs injected with coal-mine dust and bacilli yielded growth of tubercle bacilli up to, but not beyond, 120 days. The largest number of organisms was found at 30 days. In animals infected with bacilli alone no growth could be obtained from the lung cultures except from the guinea-pig killed one day after the intratracheal injection.

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STUDIES OF THE DEVELOPING TUBERCULOUS LESION BY PHASE CONTRAST MICROSCOPY.

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THE development and progress of tuberculous lesions have been extensively studied by various histological techniques and are now well known. In recent years attempts have been made to study these more intimately by methods which would reveal the detailed processes at reasonably high magnifications. One method which has proved useful is that of the rabbit ear chamber (Ebert, 1952) in which the progress of a lesion can be studied *in situ* from day to day at magnifications up to about × 400. Though this is a useful technique it does not reveal intimate details of cellular and intracellular changes. Another method which has yielded very interesting results is that devised by Mackaness (1952), who studied *in vitro* the fate of macrophages containing tubercle bacilli of various strains and the effects of drugs on the process.

We were interested in investigating in detail the progress of tuberculous lesions in the cornea, particularly in the days after inoculation during which no changes can be seen with the naked eye or under the binocular microscope at magnifications up to about × 20. We hoped to devise a method of observing these changes *in situ* in the live animal from day to day with high magnifications, using the phase contrast technique, but so far we have failed. We have, however, found that when the cornea is removed from mice and placed between a slide and coverslip with a drop of saline, its intimate structure can be clearly seen at high magnifications and the details of infective processes stand clearly revealed ; it is by this method, using phase contrast microscopy, that we have followed, from day to day, the development of the tuberculous process in the cornea of mice.

METHODS.

Using the technique described by Rees and Robson (1950), albino mice were inoculated intra-corneally with dilutions of a Dubos culture of a bovine strain of *Mycobacterium tuberculosis* (originally supplied by Dr. Levinthal), whose virulence in mice was known to be high. At various intervals after inoculation animals were killed and the infected eye removed. Occasionally, and particularly in the early stages of this work, the other (normal) eye was used as a control where there was any doubt about the nature of some structure seen.

The cornea was removed from the eye as a small cup and separated from the lens and iris. Two or three radial cuts were made which allowed the cornea to collapse as a flat sheet. This preparation was mounted between a glass slide and coverslip in normal saline, usually with its posterior surface uppermost (since lesions initially develop in the posterior part of the cornea). Surface tension of the saline caused a further flattening of the cornea, and serious creasing was not encountered. The preparation was kept at room temperature in a warm stage containing water in a watch glass to prevent evaporation. Mounting in glycerine mixtures rendered much fine structure invisible, presumably due to the increased refractive index of this medium, and could therefore not be used.

A binocular Cook microscope with standard × 10, × 40, × 95 phase contrast objectives and phase plate, with × 8 eye-pieces and a calibrated measuring graticule were used throughout.

Before dissection eyes were examined by reflected light with a low power binocular microscope to determine whether any lesion was visible. In some cases eyes have been removed for histological examination in order to compare such findings with the appearances seen by phase contrast. After removal the whole eye was fixed for at least 24 hr. in Bouin's solution. The whole cornea was then cut out, dehydrated, cleared and embedded in paraffin. Sections, 4–5 μ in thickness, were cut along the plane of the cornea and mounted in such a way that corresponding material was available for staining with either haematoxylin and eosin, or by the Ziehl-Neelsen method (counterstained with methylene blue).

RESULTS.

Normal Eyes.

Viewed macroscopically by reflected light a normal mouse cornea appears as a transparent hemisphere sometimes with a barely perceptible sheen on it. With phase contrast examination its full structure is well seen when prepared as described above. Focusing down from the posterior surface the following structures can be seen :

(1) An endothelium of cells whose nuclei are about 10 μ in diameter and sometimes rather square. These cells appear to be intermixed with an oily deposit which is seen as light structureless globules. The layer is very thin.

(2) The main corneal substance, which is an almost acellular space, some 50 μ in depth. In this region cells do occur (corneal corpuscles) but are few in number. Nerves are seen as wandering tubes with nuclei at intervals along them. The nerves usually start in groups of about 6 near the limbus and diverge towards the centre, becoming thinner as they go.

(3) Next come two or three organised layers of closely packed small cells.

(4) Finally, there is the anterior epithelium, which consists of typical cells some 30 μ in diameter with nuclei about 8 μ in diameter.

A considerable amount of detail (especially of nuclei) is visible at all levels, but the upper (posterior) parts are clearest. It will be seen that the corneal substance is almost free from visible structure, and this is a great advantage since it is here that the organisms are deposited and the lesion develops.

Normal Course of Infection.

In previous work on corneal tuberculosis (Rees and Robson, 1950) a suspension containing about 1,000 organisms in 0·01 ml. was injected into the cornea. This produces a bleb which disappears within 24 hr. and for a latent period of about 11 days the cornea is then entirely clear on macroscopic examination under the binocular microscope. At the end of the latent period minute lesions become visible, which then rapidly spread. We decided to introduce more bacilli in order to make microscopic observation easier and hence ten times the number of bacilli was introduced, *i.e.*, about 10,000 organisms in the usual amount of 0·01 ml. of 0·05 per cent Tween saline. With this larger inoculum the latent period is decreased, but even so, after the initial bleb no lesion is usually visible macroscopically for about 7–8 days and we were particularly interested to see what happened in the cornea during this latent period.

When the material is injected into the cornea an area of opacity is formed; this bleb covers about one-third of the cornea and sometimes more, and before this work was undertaken we thought that the organisms were distributed over the area of the bleb. When such a bleb is examined by phase contrast a few hours after inoculation it is found that the organisms are restricted to a small area (about 500 μ in diameter) which corresponds roughly to the tip of the needle. It would appear that on intra-corneal injection bacilli suspended in the injected medium are filtered out almost immediately at the tip of the needle by the corneal substance so that their area of distribution corresponds roughly to the needle tip and very few bacilli spread further. Shortly after injection, therefore, the organisms can be seen around the mark left by the needle tip, which appears as a triangular distorted area. The organisms are visible as single rods, in small clumps, and also occasionally in very large masses, each of which may contain hundreds of organisms. The bacteria appear as short quite dark rods, whose size is normal for this stage of growth of *Myco. tuberculosis*. At this stage, within a few hours of the inoculation, there is no cellular invasion, and the only abnormality is a tissue disruption due to the entry of the needle and the deposition of bacilli around its tip (Fig. 1.).

In view of the importance of the site of distribution of the organisms, an additional experiment was performed to establish this beyond doubt. Stained organisms were injected into the cornea so that their identification could be made with certainty and be independent of the phase contrast technique. The organisms were stained as follows:

A known volume of a 14-day Dubos culture of the same strain as that already used was centrifuged for 10 min. at 5000 r.p.m., which deposited all the organisms. The supernatant fluid was removed and replaced by the same volume of filtered carbol fuchsin containing 0·1 per cent Tween. The organisms were re-suspended by shaking and the suspension immersed in a water bath at 80° for 30 min. Centrifugation as above was repeated and the carbol fuchsin removed. The bacilli were washed six times with a 0·1 per cent Tween saline. In spite of the use of Tween, clumping of the organisms was very marked; incubation at 37° overnight produced a well separated suspension. Before use the organisms were re-immersed in a water bath at 80° for 30 min. to kill them, and the 0·1 per cent Tween saline was replaced with 0·05 per cent Tween saline by centrifugation and re-suspension of the organisms.

When such a suspension of stained organisms was injected into the cornea at a dilution equivalent to 1/100 of the original culture (as used in the experiment described above) and examined with ordinary transmitted light the area of distribution was found to be restricted to the needle tip, as seen by phase contrast. Even when 50 times as many stained organisms were injected this was still found to be the case, thus confirming that the organisms are effectively filtered out by the cornea from the suspension and, incidentally, establishing the validity of the phase contrast technique.

Development of the lesion.

By 24 hr. after inoculation considerable changes have taken place. An area roughly equivalent to that in which the organisms were deposited is filled with a complex mass presumably consisting of invading cells. This is several layers in thickness and detail within it cannot be resolved. Very few or no organisms are seen at the periphery of this mass. In addition the whole cornea contains considerable numbers of small invading cells whose diameter is 10–12 μ and which appear

grey on phase contrast examination. At this stage the whole cornea is completely clear when observed in the intact animal through a dissecting microscope.

A striking feature at this and later stages is the presence of a large number of minute spherical masses of unknown nature about 0·25–1·25 μ diameter (Fig. 2). The distribution of these dots is not entirely random and in places they appear in an orderly pattern of which the significance is unknown. Their presence can be very helpful in locating the lesion.

We have been puzzled by our failure to see more than a few organisms in and around the central mass at this stage. It is possible that they are masked by the thick central core, though this explanation is unconvincing in view of the detection of a large number of organisms at a later stage of the lesion. It is also possible that the organisms are present but engulfed by leucocytes in which, for some reason, they are not visible by phase contrast.

Examination of sections of eyes at this stage showed clearly that the central lesion consists essentially of numerous polymorph leucocytes, though a few macrophages can also be seen. A few invading leucocytes can also be identified in the part of the cornea surrounding the lesion.

Organisms were also identified. They were not numerous and those seen were all extracellular : some were acid-fast, and in some of these dark polar bodies were seen at one or both ends. A few such bodies were seen apparently independent of any bacillary part, though they may have been bacilli lying perpendicular to the plane of examination. A few organisms were also seen which did not stain red but appeared rather bluish and ghost-like. Such organisms were, however, also seen in Ziehl-Neelsen preparations of material similar to that used for inoculation. Nothing corresponding to the dots was seen in any of the material examined histologically (though no special examinations, *e.g.*, with other stains or on frozen sections, have so far been made).

By *two days* after inoculation the position had not greatly changed, the main alteration being that the lesion is now surrounded by some macrophages which however do not contain bacilli but only dots. There is no significant change in the size of the lesion.

Sections at this stage showed that in the central lesion macrophages predominated though many polymorphs were still present. Search of many sections obtained from four eyes (from different animals) revealed the presence of two organisms (found extracellularly). Hence few organisms can be identified 24 hr. after inoculation and they have practically disappeared one day later. These findings were consistent both by the usual histological methods and by phase contrast.

At *three days* after inoculation some macrophages contain bacilli and a few are packed with them (Fig. 3), but many appear quite normal and no organisms are visible within them. The remainder of the cornea is now essentially free from all invading cells and is quite normal in appearance. Again there is no increase in the size of the lesion which is still not visible macroscopically.

Five days after inoculation there is still little change in the size of the lesion, but it has obviously progressed. Macrophages and smaller grey cells surround the lesion, with many of the former full of bacilli. In some cases the macrophages are obviously beginning to break down under the stress of the dividing bacilli in the same way as has been described by Mackaness in his work *in vitro* on rabbit macrophages. In others only masses of débris in connection with large numbers of

bacilli arranged in a definite pattern are evidence that macrophages have fully broken down, releasing their contents *in situ* (Fig. 4).

When the bacilli are present in large numbers in macrophages or in masses subsequent to the breakdown of the macrophages they are seen as long thin rods almost filamentous in appearance whereas before this stage they are shorter and thicker. This is quite a striking and consistent phenomenon and in keeping with our knowledge of the natural history of the organism.

By the *7th–8th day* a lesion is seen macroscopically. Microscopically it consists of the central core as previously described, but now the lesion is obviously enlarging and the spread is by finger-like processes. These consist of chains of macrophages mostly in an advanced stage of breakdown, as is illustrated in Fig. 5. These processes are full of long and well-defined bacilli and also contain a large number of dots.

The spread of the infection is not due solely to the activity of macrophages since chains of bacilli have been observed beyond the confines of the lesion; these are often seen beyond the tip of one of the finger-like processes. The bacilli are all definitely orientated, as is illustrated in Fig. 6. They appear to be present along the walls of minute canals some 2μ in diameter and these may spread well beyond the edge of the lesion. These canals may be minute nerves and the perineural spaces, but this requires further investigation and confirmation, as does the possibility that the finger-like processes observed as evidence of the spread of the lesion originate by spreading along canals, the organisms then subsequently becoming engulfed by macrophages.

DISCUSSION.

By this simple method it has thus been possible to follow in detail from its inception the development of a tuberculous lesion. The cornea is rapidly scanned in three dimensions at fairly low magnification (× 120) and when the main focus is localised more detailed examination at higher magnification (× 1200) easily follows. Hence in a relatively short time it is possible to obtain information which the usual histological methods only reveal after much time and labour. The site of deposition of organisms, the early cellular invasion, and the subsequent processes involving multiplication of bacteria within macrophages with their ultimate disruption are clearly seen. The correlation between the morphology of the organisms and the stage of development of the lesion is also clear, *i.e.*, the short almost

EXPLANATION OF PLATES.

FIG. 1.—*Myco. tuberculosis* in the cornea 3 hr. after inoculation. Phase contrast × 2,500.

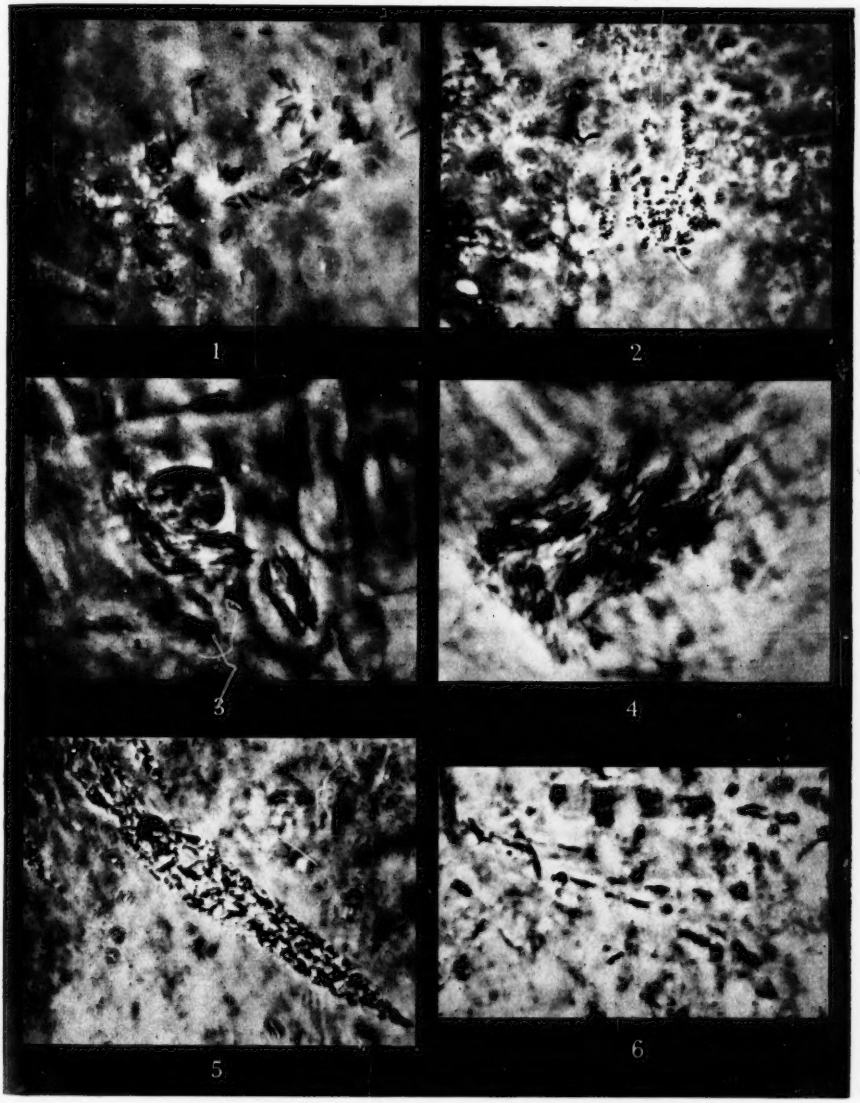
FIG. 2.—Extracellular dots in the cornea, of the type frequently seen associated with lesions. Phase contrast × 1,200.

FIG. 3.—Macrophage containing large numbers of long bacilli. The cell is obviously still in reasonably healthy condition as seen from the appearance of its nucleus. Phase contrast × 3,000

FIG. 4.—Numerous bacilli, almost filiform in appearance; the general configuration of the mass suggests that the bacilli occupy the site of breakdown of one or more macrophages. Phase contrast × 3,000.

FIG. 5.—"Finger-like" process extending from a spreading lesion, 7 days after inoculation. Note numerous long bacilli, dots and débris. Phase contrast × 1,400.

FIG. 6.—Extracellular bacilli in the cornea at some distance from the main focus of a lesion, 12 days after inoculation. Many of the bacilli appear orientated along a canal. Phase contrast × 4,500.



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coccoid form in the early stages progressing ultimately to the almost filamentous appearance in the breaking down macrophages. It is well realised that it would be even more satisfactory to be able to follow the process in one and the same animal, but unfortunately this has not so far proved possible.

The findings so far discussed are in good agreement with those previously obtained with other methods. Not only do they confirm the information obtained from histological investigations but they also demonstrate in the tissue what Mackaness (1952) has shown in infected macrophage culture, *i.e.*, the development of *Myco. tuberculosis* within the host cell to the point of its ultimate destruction.

Some additional findings seem worthy of discussion, in particular the method by which the disease process spreads from its original focus. When we first observed the finger-like processes extending from the periphery of the lesion we believed them to be chains of infected macrophages which had migrated from the lesion. That this is not the only process involved is strongly suggested by the finding of extracellular bacilli at some distance from the lesion and from any cells : we have done our best to exclude the possibility that they were deposited there at the time of the inoculation ; even had this occurred it seems highly unlikely that they would have remained extracellular all this time. Moreover these bacilli were frequently seen orientated in one direction, sometimes along some kind of channel, suggesting extracellular movement beyond the periphery of the lesion perhaps passively under the stress of mechanical forces, or even by some process at present unknown.

Other points which require further investigation are the disappearance of the organisms soon after inoculation, and the presence of dots for which no histological counterpart has so far been found.

It seems to us that this technique is well suited for the investigation of various aspects of tuberculous and other infections, and for studying the action of drugs. A number of such investigations are already in progress.

SUMMARY.

A method is described for the examination of the excised cornea by phase contrast microscopy ; the cornea is inoculated with *Myco. tuberculosis* and removed from different animals at various stages after inoculation. Within a few hours of inoculation the organisms can be seen over an area about 500 μ in diameter. The subsequent cellular invasion by leucocytes and macrophages can be followed. Later, the organisms proliferate within the macrophages, which then break down, and the lesion spreads by finger-like processes. Organisms can also be seen extracellularly well outside the main lesion, apparently spreading along canals.

We are greatly indebted to Dr. M. Naguib for inoculating a number of the mice used.

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STUDIES ON THE PATHOGENICITY OF *STREPTOCOCCUS PYOGENES*. III: THE UTILISATION OF ^{59}Fe, ^{65}Zn and ^{32}P BY GROUP A STREPTOCOCCUS AND THE QUANTITATIVE DETERMINATION OF THE AIR-BORNE INFECTION OF MICE WITH "TAGGED" CELLS.

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THE studies of Coburn, Evans and Nathan (1954) have shown that fatal airborne infections can be induced in Swiss albino mice by aerosol spray of group A streptococci. It was pointed out that respiratory tract infections could be accomplished with certain strains of cocci; other strains of the same serological type were found incapable of causing fatal infection in the mouse *viâ* an aerosol spray. The infective strains were classified as mouse virulent or avirulent as determined by the intraperitoneal injection of suspensions of each strain. Irrespective of type or strain, the streptococci were inhaled under the experimental conditions described. The number of organisms taken in was unknown.

The present study is concerned with establishing the aerosol technique on a quantitative basis. On this basis, the number of micro-organisms inhaled per mouse under the influences of the daily variations in procedure can be closely evaluated. This permits the determination of any differences in the numbers of bacterial cells taken in by mice when exposed to an aerosol spray. If there are differences in the numbers of cocci of infective or non-infective strains inhaled, these differences can be detected by quantitative studies.

Goldberg and Leif (1950) and Liao and Krehl (1951) have shown that bacterial cells can be "tagged" with ^{32}P. With the aid of the defined medium of Slade and Slamp (1955) for the growth of *Streptococcus pyogenes*, group A haemolytic streptococci labelled with ^{65}Zn, ^{59}Fe and ^{32}P have been obtained. Data will be presented which show that approximately the same number of cocci are taken in per mouse under the conditions used by Coburn *et al.* (1954). No differences due to strain or type could be demonstrated. A method is presented for the accurate enumeration of the total number of streptococci in an aqueous suspension, and data are given on the uptake of ^{59}Fe, ^{65}Zn and ^{32}P by several strains of *Str. pyogenes*.

MATERIALS AND METHODS.

Culture of organisms.

All strains were grown in the defined medium and under the conditions described by Slade and Slamp (1955). The iron, zinc or phosphate salts were omitted from the basal salt mixture in order to avoid dilution of the isotope on addition of the isotope to the medium. Phosphate, which also serves as a buffer in the medium, was replaced by 80 mg. sodium acetate and 80 mg. sodium carbonate. The growth response of type 19 haemolytic streptococcus (strain N19) to the addition of phosphorus under these conditions is illustrated in Fig. 1.

The cells were harvested by centrifugation, washed three times with sterile distilled water equal in volume to that of the broth and re-suspended in sterile distilled water. An aliquot was removed and determination made of isotope content. All counts are expressed as counts/minute (c.p.m.).

The isotopes, purchased on allocation from the Oak Ridge National Laboratory, possessed the following specific activities in μc/g.: ^{59}Fe, 1100–1500; ^{65}Zn, 85–125; and ^{32}P, 40,000.

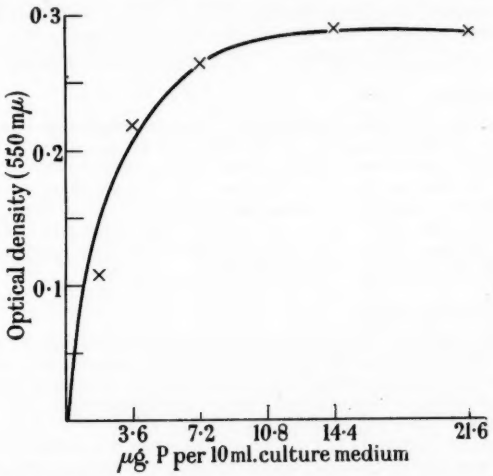

FIG. 1.—The growth response of strain N19 to varying quantities of phosphorus (added as K_2HPO_4). Culture incubated 18 hr. at 37°.

Aerosol technique.

The procedure used was similar to that of Coburn *et al.* (1954). A measured quantity (about 5 ml.) of standardised cell suspension was sprayed into the chamber over a period of 25–30 min. The rate of air flow was about 5 l./min. At the end of the spraying period the cells remaining in the nebuliser were washed into a volumetric flask and diluted to the mark. Isotope counts were made on the original cell suspension (after adequate dilution in water) and the residual remaining in the nebuliser. The difference between these two counts represented the quantity of isotope sprayed into the chamber.

Isotope counts on mice.

At least 5 mice (generally 6) were used for each experiment. At the conclusion of the spraying period, the mice were held in the cage for 30 min. They were then removed, sacrificed by etherisation and washed thoroughly under a stream of running tap water. The fur of the animal was rubbed back and forth during the washing procedure to insure the removal of the labelled cocci. Previous experiments with both living (muzzled) and dead mice had shown that this procedure was satisfactory. The ^{32}P count of these animals after washing was reduced to 10 per cent or less that of control animals.

The entire animal was carved into pieces and slowly dried in an evaporating dish over an open flame for about 8 hr. The residue was then ashed in a muffle furnace at 700°. The ash was refluxed in 6 N-HCl for 30 min., adjusted to a known volume and filtered. An aliquot was transferred to a beaker, evaporated to dryness, taken up in a small volume of water and transferred to a planchet. The water was evaporated in a stream of air on a warm plate, the washings from the beaker added to the planchet and the evaporation repeated. All isotope counts were made in a flow counter. All animals were handled separately and the isotope counts averaged for each experiment.

Isotope counts on air samples.

Samples of air (40 ml.) were withdrawn at various times following the spray period from the interior of the aerosol chamber through a needle and into a 50-ml. glass syringe which contained 10 ml. water. The needle was inserted through a rubber stopper. The needle and stopper had been inserted in place of the atomiser in the wall of the compartment at the conclusion of the spray period. The needle was not removed from this position during the sampling period.

The syringe was shaken frequently during the 2 hr. following the withdrawal of the air sample. The water was removed and the interior of the syringe rinsed several times. The water plus rinses was adjusted to a known volume and a sample removed for isotope count.

Enumeration of streptococci.

An accurate procedure for the enumeration of the streptococcus is essential in order to determine the number of cells in relation to the isotope content of the mice. The counting of bacteria in the Petroff-Hausser chamber depends on an even distribution of single cells throughout the chamber. It is obvious that the tendency of most streptococci to form chains and clumps presents a serious problem in quantitative work. This difficulty was overcome by exposure of cell suspensions to a 2-min. period of sonic oscillation under the conditions previously described (Slade, 1953). By this procedure the cells were separated into single units and reproducible counts were readily obtained.

Standardisation of cell suspensions.

It next became necessary to determine what differences, if any, existed among various strains in the number of cocci per ml. present in an optically standardised suspension. Although optical methods have been used for some years to measure the growth of bacteria, the procedure had not been checked with various strains of group A streptococci.

Washed cells were re-suspended in water by shaking briefly by hand. The suspension was standardised to an optical density (O.D.) of 0·65 at 550 mμ and oscillated for 2 min. An aliquot was then counted in the Petroff-Hausser counting chamber under a magnification of × 430. Table I shows the results obtained. Under the conditions of these experiments and for the strains examined, standardisation to an O.D. of 0·65 resulted in a suspension of streptococci that contained between 24·0–26·4 × 10^9 cells/ml. In this study a figure of 25 has been used in all calculations.

TABLE I.—*Standardisation of Cell Suspensions of Group A Streptococci by Optical Density.*

Culture.		
Strain.	Type.	Cocci. per ml. × 10^9.
N19	19	25·2
T19	19	26·4
IGL23	17	25·2
S43	6	24·4
Richards	3	24·0

Washed cells diluted to O.D. 0·65 (550 mμ.) oscillated at 9 kc. for 1 min. at 2° and counted in Petroff-Hausser counting chamber.

RESULTS.

The data of Tables II, III and IV illustrate the uptake of ^{32}P, ^{59}Fe and ^{65}Zn by four strains of group A streptococcus, representing three serological types. The per cent uptake of isotope per 10^{10} cells (column 5 in each case) shows that strains T19 and 12RN incorporated more Fe, Zn and P into the cell than either of the other two strains. The difference between strains ranged from twofold with P to about fifteenfold with Zn. The quantity of P taken up by all strains was considerably greater than that of Zn (10–250 times) and somewhat greater than that of Fe (5–70 times.)

The calculations of cells/10^3 c.p.m. (column 6, Tables II, III and IV) show clearly that the cells with the highest isotope content were those grown in the presence of P. This is due not only to a greater uptake, as pointed out above, but also to the higher isotope content of the phosphorus as indicated by its specific activity. In the aerosol experiments reported here cells with a high isotope content are to be preferred. In brief, ^{32}P proved to be the best isotope for the purposes of this study.

TABLE II.—*The Incorporation of ^{32}P into Cells of Group A Streptococci.*

Strain.	Tube no.	Optical density.	C.p.m. taken up $\times 10^5$.	Per cent uptake per 10^{10} cells.	Cells per 10^3 c.p.m.
N19, type 19	1*	0·18	274	9·3	2·6
	2*	0·19	285	9·8	2·5
T19, type 19	3† (2 tubes combined)	0·68	244	26·1	10·7
S43, type 6	4*	0·20	362	13·1	2·1
	5*	0·21	332	13·1	2·4
12RN, type 12	6*	0·41	362	27·2	4·3
	7*	0·37	313	21·2	4·4

C.p.m. of culture fluid before inoculation : * 207 $\times$ 10^4 ; † 244 $\times$ 10^4.

TABLE III.—*The Incorporation of ^{59}Fe into Cells of Group A Streptococci.*

Strain.	Tube no.	Optical density.	C.p.m. taken up $\times 10^5$.	Per cent uptake per 10^{10} cells.	Cells per 10^3 c.p.m.
N19, type 19	1*	0·27	7·5	2·3	138
	2*	0·31	5·5	1·9	216
T19, type 19	3*	0·36	16·0	6·3	86
	4*	0·34	13·0	5·0	101
S43, type 6	5*	0·22	2·2	0·5	380
	6*	0·20	1·8	0·4	450
12RN, type 12	7† (2 tubes combined)	0·60	19·5	3·7	119

C.p.m. of culture fluid before inoculation : * 346 $\times$ 10^4 ; † 123 $\times$ 10^4.

TABLE IV.—*The Incorporation of ^{65}Zn into Cells of Group A Streptococci.*

Strain.	Tube no.	Optical density.	C.p.m. taken up $\times 10^5$.	Per cent uptake per 10^{10} cells.	Cells per 10^3 c.p.m.
N19, type 19	1*	0·16	1·2	0·2	502
	2*	0·17	1·5	0·3	444
T19, type 19	3*	0·28	5·2	1·6	200
	4*	0·24	4·7	1·3	194
S43, type 6	5*	0·18	0·5	0·1	1360
	6*	0·15	0·5	0·1	1170
12RN, type 12	7† (2 tubes combined)	0·53	4·5	1·7	400

C.p.m. of culture fluid before inoculation : * 337 $\times$ 10^4 ; † 540 $\times$ 10^4.

TABLE V.—*Aerosol Infection of Mice with ^{59}Fe-, ^{65}Zn- and ^{32}P-"Tagged" Streptococci.*

Isotope used.	Culture. Strain.	Type.	Cells sprayed in, × 10^9.	Isotope sprayed in, c.p.m. × 10^6.	Isotope per mouse, c.p.m.	Per cent isotope per mouse.	Total cocci per mouse, × 10^4.
^{59}Fe	N19	19	137·5	7·8	910	0·12	160
	T19	19	137·5	6·2	600	0·09	133
	12RN	12	137·5	48	5,360	0·10	153
	S43	6	137·5	21	1,725	0·08	113
^{32}P	B19	19	132·5	371	34,800	0·08	124
	N19	19	150	217	16,700	0·11	116
	123389	5	125	24	2,900	0·12	151
^{65}Zn	N19	19	150	60	4,000	0·07	100

Table V presents the complete data on eight experiments in which labelled streptococci were used to infect mice *viâ* an aerosol spray. Column 7 shows that approximately the same number of cocci were taken in per mouse, the overall range being less than twofold. These experiments were made over a period of 6 months (December to May) and are believed to illustrate clearly the range in number which can reasonably be expected to occur under changing laboratory conditions; for example, fluctuations in temperature, humidity and atmospheric pressure. It is clear that the technique of aerosol infection used resulted in a uniform quantitative infection in the mice. It is also apparent that there were no significant differences in the number of cells taken in between the several serological types or between the strains within a type examined in this study.

The uniform degree of infection obtained indicated that the "fall out" or settling down of the moisture droplets containing the cocci, followed a similar pattern among the strains tested. A marked dissimilarity in the rate of "fall out" would probably have resulted in greater differences than were observed. Fig. 2 shows that when two strains of group A haemolytic streptococcus (N19 and 12RN) were sprayed into the compartment in approximately the same number, the rate of "fall out" of the tagged cells was similar in both cases. The bacterial count of the air after 10 min. was about 70 per cent of the value at the time when the circulating fan was turned off; after 15 min. the value was about 60 per cent; thereafter the decrease was at a slower rate.

DISCUSSION.

The use of streptococci tagged with Fe, Zn or P isotopes has provided a convenient and reliable means for the accurate determination of the inhalation of streptococci by mice. The experiment must be of short duration (3 hours or less). Otherwise, the elimination of the isotope in the faeces, urine, etc., will lower the bacterial count of the animal as calculated from the isotope content. The data show that the aerosol procedure used (1 hour elapsed time) resulted in a strikingly uniform degree of infection regardless of the strain or type of group A haemolytic streptococcus used.

Sonic oscillation has been shown to be an important aid in the accurate enumeration of streptococci. In addition to facilitating the microscopic cell count,

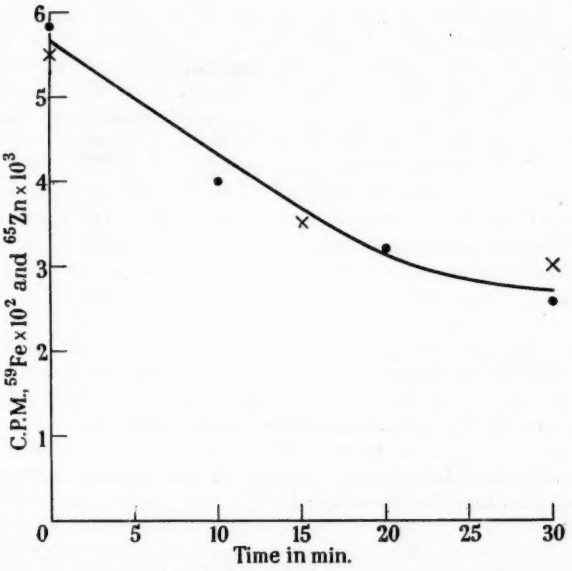

FIG. 2.—The " fall out " of moisture droplets containing ^{59}Fe and ^{65}Zn-labelled streptococci. Solid circles, strain 12RN, each c.p.m. represents $2 \cdot 9 \times 10^4$ cells ; crosses, strain N19, each c.p.m. represents $2 \cdot 5 \times 10^4$ cells.

it has been found to increase the living cell count (as judged by pour plate procedure) from 2–5 times (Slade, unpublished results). The reason for the apparent increase in living cells is evidently due to a break-up of the chains and clumps of cells so that individual cells have an increased opportunity to produce a single colony in the agar.

The comparative differences in the amount of iron, zinc and phosphorus taken up by the several strains reflects the requirements of these ions for the synthesis of the numerous components of cell protoplasm. The need for phosphorus in the synthesis of nucleoproteins and possibly other phosphorus-containing proteins, which make up a large part of bacterial cell substance, is shown by the striking uptake of this element.

SUMMARY.

Data are presented on the uptake of ^{65}Zn, ^{59}Fe and ^{32}P from a defined medium during the growth of four strains (three serological types) of group A haemolytic streptococcus. The differences between strains as to the quantity of isotope incorporated into a constant number of cells ranged from twofold with P, tenfold with Fe and fifteenfold with Zn. The quantity of P taken up by a constant number of cells was considerably greater than the quantity of the other two elements.

Aqueous suspensions of streptococci can be accurately counted in a microscopic counting chamber after a brief exposure to sonic oscillation. Oscillation breaks up chains and cell clumps, giving an even distribution of cells throughout

the chamber. Suspensions of various strains of group A cocci (optical density 0·65, 550 mμ.) contained between 24·0–26·4 × 10^9 cells/ml.

By the use of " tagged " cocci, it is demonstrated that under the experimental conditions described, the number of bacteria taken in by the mouse was approximately 130 × 10^6 cells. The variation in the average number of cocci taken in per animal in each experiment was less than twofold. This was true for all strains tested during the six-months period of this study.

The findings indicate that the critical factor in the pathogenicity of certain strains of group A haemolytic streptococcus for Swiss albino mice, as determined by the air-borne induction of fatal respiratory tract infections, is neither the number of micro-organisms in the air nor the number of micro-organisms inhaled by the animal.

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THE BIOLOGICAL ASSAY OF MYXOMA VIRUS.

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TITRATIONS of viruses have almost invariably been made in terms of their infectivity, although haemagglutination is also used in the titration of influenza virus. Infectivity is usually determined by the inoculation of groups of animals with serial dilutions of the preparations under study, presence or absence of the virus being indicated by the presence or absence of a clearly observable end-result, often death. End-points derived from such experiments are usually expressed as a 50 per cent infective dose (ID_{50}). With some viruses pock counting methods on the chorio-allantoic membrane of the developing egg have been employed (Beveridge and Burnet, 1946), and more recently the same principle has been applied to plaques produced on tissue culture monolayers (Dulbecco and Vogt, 1954).

With vaccinia and myxoma viruses a variation of the quantal response method has been used, namely the intradermal inoculation of the rabbit skin at many sites with replicates of several dilutions of the preparation under examination, the development of a skin lesion at the inoculation site indicating a positive response (Parker, 1938 ; 1940). The infectivity of these viruses can also be titrated on the chorio-allantoic membrane of the developing chick embryo (Keogh, 1936 ; Lush, 1937). Fenner and his collaborators (Fenner and Woodroofe, 1953 ; Fenner, personal communication), have recently compared the sensitivity and accuracy of these two methods of titration.

Both these methods demand for their accurate performance considerable numbers of experimental animals. It occurred to us that myxoma virus might be assayed by the methods commonly used in the assay of pharmacological preparations, namely the measurement of the diameter of lesions produced by the intradermal inoculation of unknown and standard preparations of the virus in the rabbit.

MATERIALS AND METHODS.

Myxoma virus.—Our thanks are due to Professor F. Fenner for supplies of standard and Lausanne strains of virus and to the Veterinary Research Institute, Glenfield, for the Glenfield strain. The virus was stored in sealed glass ampoules on dry ice and thawed immediately before use. Thawed virus provides the undiluted (10°) suspension for injection.

Rabbits.—Forty-six domestic rabbits were used in the investigation. The 42 rabbits used in the preliminary experiment were housed at the Animal Genetics Section Rabbit House, Prospect, without temperature control. The remaining 4 were housed at the Department of Veterinary Physiology in a room thermostatically controlled at 75° F.

Before use in an assay the back of the rabbit was closely clipped and marked with picric acid in a grid to form a 2 × 2, 6 × 6 or an 8 × 8 chess board pattern. Intradermal injections of 0·1 ml. virus in distilled water were made to the centre of the appropriate squares.

After 4 or 5 days the diameter of each lesion was measured to the nearest mm. with a pair of calipers. The diameter was measured at the edge of the raised indurated portion of the

lesion and the remaining rather variable inflamed area disregarded. In the preliminary experiment the animals were observed at 6-hr. intervals for the occurrence of death.

Mice.—Eighteen randomly bred new-born mice of Agouti parents were employed. Mice were inoculated intracerebrally with 0·02 ml. of 10° Glenfield strain virus by means of an Agla micrometer syringe and 27-gauge hypodermic needle. At the appropriate time three mice were killed by decapitation and the heads, together with 6 ml. penicillin solution (50,000 u./ml.), were ground in a mortar with carborundum grit. Periodically during grinding the mortar and contents were placed in the deep freeze (− 20°) to provide alternate freezing and thawing and thus break down cells. The resultant suspensions were centrifuged at 2500 r.p.m. for 15 min. and the supernatant stored in glass ampoules on dry ice prior to testing.

RESULTS.

Preliminary experiment.

While it has been suspected that both killing time and lesion size are a function of dose of virus they have not been used as a means of assay. The prime object of the preliminary experiment was to determine the nature of this relationship in three virulent virus strains—standard strain (S.S.), Lausanne strain (L.S.) and Glenfield strain (G.S.). The effect of artificial spreading of virus by injection with air was also studied in this experiment. One half of the rabbits received the virus suspension intradermally, 0·25 of the dose being inoculated to each of four different sites in the standard manner, while the other rabbits received the same treatment together with 0·5 ml. air injected at each site to promote spreading. Seven different doses (4×10^{0}, 4×10^{-1}, 4×10^{-2}, 4×10^{-3}, 4×10^{-4}, 4×10^{-5}, 4×10^{-6}) were chosen for study for each strain and under both injection treatments, the whole experiment forming a $3 \times 2 \times 7$ factorial design. The design and results are shown fully in Table I.

TABLE I.—*The Responses of 42 Rabbits to the Treatment Combinations Described in the Text.*

		Killing time (6 hr.). Dose.						
Strain.	Treatment.	4×10^{0}.	4×10^{-1}.	4×10^{-2}.	4×10^{-3}.	4×10^{-4}.	4×10^{-5}.	4×10^{-6}.
G.S.	Standard	40	41	42	44	44	44	52
	Air	40	40	56	42	44	48	48
S.S.	Standard	40	40	42	41	40	41	50
	Air	36	40	47	40	40	44	40
L.S.	Standard	49	43	40	44	48	48	49
	Air	36	42	44	44	48	52	52
		Lesion diameter (mean mm.).						
G.S.	Standard	25	26	20	18	10	1	4
	Air	29	32	26	23	19	14	17
S.S.	Standard	24	22	21	19	17	19	1
	Air	28	26	23	26	21	19	17
L.S.	Standard	27	23	23	24	15	10	1
	Air	28	25	18	19	12	15	5

The observations have been reduced by standard analysis of variance procedures (see Kempthorne, 1952) the results of which are presented in Table II. The logarithm to the base 10 of the killing time measured in 6-hr. intervals (to preserve

TABLE II.—*Analysis of Variance of the Data of Table I.*

Source of variation.	D.f.	Mean square. Killing time.	Mean square. Lesion diameter.
Virus strains	(2)		
S.S. *v.* L.S. + G.S.	1	1·26**	46
L.S. *v.* G.S.	1	0·07	14
Treatment	1	0·00	201**
Doses	(6)		
Linear	1	2·41***	1716***
Remainder	5	0·11	15
Interactions as error	32	0·129	16·8

** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.

whole numbers) was employed in this analysis since it was thought that variances would be more homogeneous on this scale. Lesion size was employed untransformed in the analysis of variance.

Both analyses of variance indicate that each response is linearly related to log dose in the dose interval chosen for study. The precision of an assay is governed both by the slope of the dose response line and by the error variance. The greater the ratio of the mean square for linear regression to the error variance the greater the precision. On these grounds it is seen that assays based on lesion size are about 5·5 times as precise as those based on killing time. Multiple response data such as Table I may be combined into a single index of response by discriminant analysis (see Finney, 1952*a*; Rao, 1954 for relevant discussion), but it is evident that killing time would receive little weight in such an analysis. For this and other reasons more fully discussed below it was decided to concentrate on lesion size as an assay response.

While the Lausanne and Glenfield strains may be considered as of equal potency on the basis of this preliminary work, the responses indicate that the standard strain is more potent than either of them. The effect only reaches significance with killing times, but the mean square is approaching significance with lesion diameter.

Injection of air with the virus does not have any effect on the killing time although it affects the size of the lesion. Injection of 0·5 ml. air with each viral injection gives a lesion size equivalent to a multiplication of the dose by 23·3.

In both analyses of variance all interactions have been pooled in order to arrive at an estimate of error with a reasonable number of degrees of freedom (D.f.). This estimate of error may be regarded as between-animal variance, if it is assumed that these interactions are not significant. Since the average lesion size in Table I is based on four individual lesions obtained on the same animal, it is possible to compare the between-animal variation (0·168, 32 D.f.) with the within-animal variation 0·131, 126 D.f.) using the F test. The probability of the observed value of $F = 1{\cdot}3$ lies in the interval 0·1 –0·05, and indicates that between-animal differences contribute no more towards the variation of lesion size than would be expected on the above hypothesis.

Latin square designs.

Since the above experiment indicates that lesion size is a better response to measure than is killing time, and since many individual lesions may be initiated on one rabbit, a fully within-animal assay was designed using Latin squares to

eliminate sensitivity trends across the animal. Two assays were set up, one in an 8×8 square and the other in a 6×6 square. The Latin squares were selected at random as described by Fisher and Yates (1953). The design and analyses of variance are given in Tables III–V.

TABLE III.—*Plan of the* 8×8 *Latin Square used on the Within-animal Assay.*

The letter *S* stands for the standard preparation, *U* for the unknown preparation and the figures for different doses of each preparation as indicated below. The square of responses is in one to one correspondence with the square above.

	Transverse.							
Longitudinal.	1	2	3	4	5	6	7	8
1	*U*4	*U*2	*S*3	*S*2	*S*4	*U*3	*S*1	*U*1
2	*U*2	*U*4	*S*2	*S*3	*S*1	*U*1	*S*4	*U*3
3	*S*1	*S*4	*U*3	*U*1	*U*2	*S*3	*U*4	*S*2
4	*U*3	*U*1	*S*1	*S*4	*S*2	*U*4	*S*3	*U*2
5	*S*3	*S*2	*U*4	*U*2	*U*1	*S*1	*U*3	*S*4
6	*S*4	*S*1	*U*1	*U*3	*U*4	*S*2	*U*2	*S*3
7	*S*2	*S*3	*U*2	*U*4	*U*3	*S*4	*U*1	*S*1
8	*U*1	*U*3	*S*4	*S*1	*S*3	*U*2	*S*2	*U*4

	Lesion diameter (mm.).								Row total.
	0	10	8	10	0	0	11	13	52
	7	0	13	6	15	10	0	6	57
	17	0	7	14	11	7	0	11	67
	0	13	14	0	12	0	6	12	57
	0	10	0	8	13	10	6	0	47
	0	13	11	0	0	11	12	0	47
	10	9	9	0	0	0	11	11	50
	13	0	0	18	0	11	12	0	54
Column total	47	55	62	56	51	49	58	53	—
							Grand total		431

	Preparation.					
	Standard.			Unknown.		
Dose code.	Dose.	Mean lesion diam. (mm.).	Dose code.	Dose.	Mean lesion diam. (mm.).
*S*1	10^{a}	13·6	*U*1	$M10^{a}$	12·3
*S*2	$10^{-1.7}$	11·1	*U*2	$M10^{-1.7}$	10·0
*S*3	$10^{-3.4}$	4·5	*U*3	$M10^{-3.4}$	2·2
*S*4	$10^{-5.1}$	0·0	*U*4	$M10^{-5.1}$	0·0

TABLE IV.—*Analysis of Variance of the Data of Table III.*

Source of variation.	D.f.	Mean square.
Transverse position	7	3
Longitudinal position	7	5
Treatments	(7)	
Preparations	1	21*
Doses	(3)	
Linear	1	1688***
Quadratic	1	5
Cubic	1	57**
Parallelism	(3)	
Linear	1	2
Deviations	2	4
Error	42	5·4

* $0{\cdot}05 > P > 0{\cdot}01$; ** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.

A dilution of the standard was made by one operator and this was given to another for use in the assays, its strength being concealed from him. Tables IV and V indicate that there are no appreciable trends in sensitivity across the back of each rabbit. While the difference in potency of the two samples is only found to be significant in Table IV, Table VI shows that the relative potencies calculated from each assay are homogeneous.

TABLE V.—*Summary and Analysis of the Data Obtained in the* 6 × 6 *Latin Square Design.*

Preparations.					
Standard.			Unknown.		
Dose code.	Dose.	Mean lesion diam. (mm.).	Dose code.	Dose.	Mean lesion diam. (mm.).
*S*1	10^{0}	14·5	*U*1	$M10^{0}$	14·3
*S*2	$10^{-2.5}$	9·1	*U*2	$M10^{-2.5}$	9·1
*S*3	10^{-5}	3·8	*U*3	$M10^{-5}$	0·8

Analysis of variance.

Source of variation.	D.f.	Mean square.
Transverse positions	5	5
Longitudinal positions	5	10
Treatment	(5)	
Preparations	1	10
Doses	(2)	
Linear	1	876***
Quadratic	1	5
Parallelism	(2)	
Linear	1	12
Quadratic	1	5
Error	20	5·1

*** $P < 0·001$.

TABLE VI.—*Combination of Estimates from both Assays.*

Assay.	Slope (mm./$\log_{10}$ dose)	Relative potency (M).
8 × 8	2·70	0·37
6 × 6	2·41	0·36
Common estimate and 5 per cent fiducial limits	2·58{2·35–2·82}	0·369{0·247–0·553}
Test of homogeneity	$\chi^2_{(1)} = 1·55$; $0·3 > P > 0·2$	$\chi^2_{(1)} = 0·03$; $0·9 > P > 0·8$

Both assays indicate a strong linear relationship between lesion diameter and log dose. In the 8 × 8 assay the cubic component of regression is enlarged and on inspection of the mean values at the foot of Table III it is seen that this effect is due to the failure of response at the lowest levels of dose. It would appear that in future assays doses of less than 10^{-5}, or a comparable figure for virus produced in a different manner, should be avoided in order to preserve linearity of the dose response line. The deviation from linearity is due to the fact that below this dose (for our standard) there is a distinct probability that no lesion will develop, while above this dose the probability of development of a lesion is near 1. There is no evidence of any departure from parallelism of the dose response lines in either assay.

From each assay we may estimate the relative potency (M) of the unknown to the standard by the usual methods (Emmens, 1948 ; Finney, 1952*a*) and test the homogeneity of estimates. The relative potency depends on the difference between samples and the slope of the dose response line. In this analysis the minor cubic effect has been ignored. The slope of the dose response lines, the test of homogeneity and the fiducial limits of the common estimate are given in Table VI, together with similar statistics for relative potency.

The true value of relative potency (M) in both assays was 0·50 and the estimate obtained is in satisfactory agreement with this value.

An example of the use of the method.

Serial passage of virus through mouse brain is unlikely to lead to adaptation of the virus to this tissue unless multiplication of the virus occurs. The method of assay outlined above was used to determine the amount of virus present in the brain immediately after injection of a given dose, and one and two days after injection of the same dose to other individuals. Eighteen mice were injected intracerebrally and groups of six were killed at daily intervals. Each group was divided into two sub-groups at random and the experimental material (whole heads) pooled within sub-groups. Two dilutions were made from each of the six different unknowns to be determined and each dilution was inoculated in four sites selected at random in each of two rabbits. On each rabbit four dilutions of the Glenfield strain virus were injected at each of four sites. In all, 128 sites were inoculated, 96 with unknown material and 32 with standard material. Sites were inoculated within rabbits with no restriction to the randomisation since the previous experiment had shown Latin squares to be of no advantage. The design, results and analyses of the experiment are given in Tables VII and VIII.

The responses obtained with the unknown preparations are analysed separately from those of the standard preparation for ease in presentation. The variation in response to the unknown preparations may be broadly ascribed to two sources other than those of the treatments, namely, between-animal (subgroups of mice) variation and the smaller variation between replications of the same material on the rabbits. It will be observed that this step is exemplified on comparison of the *error* (*a*) with the *error* (*b*) using the F statistic which is very highly significant on 14 and 72 D.f. respectively. The significance of treatments must therefore be judged on the basis of *error* (*a*). In further work, therefore, it would be desirable not to replicate the same material over different sites but to use more subgroups of mice. All the D.f. of *error* (*b*) will then come into the category of *error* (*a*). An estimate of *error* (*b*) was obtained in this design for the purposes of comparison with *error* (*c*) below.

In both the standard and unknown preparations very highly significant dose effects are found. Considerable deviations from linearity are found with the standard preparation, and it is seen by inspection of the successive differences near the foot of Table VII that this is due to failure of response at the lowest level of dose. Separate analysis of the data, excluding responses to this dose level, confirms this hypothesis.

The analysis of variance shows that there is a highly significant increase in virus during the two-day period in mouse brain. This increase is clearly linear implying that equal increments of time of growth cause equal increments in lesion

TABLE VII.—*The Design and Analysis of an Assay with Six Unknown Preparations and a Standard Preparation.*

	Transverse.									
Rabbit 1.									Lesion diameter (mm.).	
	1	2	3	4	5	6	7	8		
Longitudinal										
1	*S*1	U_3^6	*S*4	U_3^1	*S*2	*S*1	U_3^5	U_3^6	16 16 0 0 15 18 9 11	
2	*S*3	*S*4	U_3^5	U_1^3	U_3^4	*S*2	U_1^6	*S*2	12 0 13 13 7 14 14 13	
3	U_3^2	U_3^2	U_1^3	U_3^2	U_1^5	U_3^4	U_1^5	U_3^2	7 10 17 9 19 8 16 0	
4	U_1^6	*S*3	U_3^5	*S*2	U_3^5	U_3^4	U_1^4	U_1^5	17 13 10 16 12 0 11 15	
5	U_1^3	U_1^3	U_1^3	U_1^1	*S*3	U_1^3	U_1^3	U_1^5	18 12 0 7 12 9 8 16	
6	*S*3	U_3^2	U_3^6	U_1^1	U_3^5	U_3^5	*S*1	U_1^6	10 0 14 7 11 14 16 17	
7	U_3^1	U_3^6	U_1^1	U_1^3	U_3^1	U_1^1	*S*4	U_1^5	0 13 8 16 0 13 4 16	
8	U_1^6	U_3^5	U_1^4	*S*1	*S*4	U_3^4	U_1^4	U_1^4	16 14 14 19 0 6 14 13	
Rabbit 2.										
1	U_1^6	U_1^2	U_3^4	U_3^3	*S*2	U_1^2	*S*4	U_1^3	18 10 6 12 12 13 0 0	
2	U_3^1	*S*1	U_1^1	*S*4	U_3^5	U_1^4	U_1^5	U_3^1	0 14 11 0 16 15 17 0	
3	U_1^5	*S*4	U_1^3	U_3^2	U_1^1	U_3^1	U_1^4	U_1^6	17 0 15 5 9 6 12 15	
4	U_3^3	*S*1	U_3^5	U_3^2	U_3^4	*S*4	U_1^1	*S*1	10 14 14 6 8 0 10 15	
5	U_3^5	U_1^4	U_3^3	*S*2	U_1^3	U_1^4	U_1^3	*S*2	11 13 11 14 10 14 12 10	
6	U_3^1	U_3^2	U_1^3	U_1^6	U_3^6	U_1^2	U_1^1	U_1^5	0 9 15 16 14 15 9 14	
7	*S*3	U_3^2	*S*3	U_1^5	*S*2	U_3^6	U_3^3	U_3^6	8 0 10 17 13 11 10 13	
8	*S*3	U_3^4	*S*1	U_1^2	U_3^6	U_3^1	*S*3	U_3^5	10 5 12 10 14 9 12 15	

Preparation.						
Standard.			Unknowns.			
Dose code.	Dose.	Mean lesion diam. (mm.).	Days of growth.	Dose code.	Dose.	Mean lesion diam. (mm.).
*S*1	10^{-1}	16 } —2	0	U_1^1, U_1^2	$M_1 10^0$, $M_2 10^0$	9, 11
*S*2	10^{-2}	14 } —3	0	U_2^1, U_2^2	$M_1 10^{-1.4}$, $M_2 10^{-1.4}$	1, 5
*S*3	10^{-3}	11 } —8	1	U_1^3, U_1^4	$M_3 10^0$, $M_4 10^0$	14, 14
S4	10^{-4}	3 }	1	U_3^3, U_3^4	$M_3 10^{-1.4}$, $M_4 10^{-1.4}$	11, 5
			2	U_1^5, U_1^6	$M_3 10^0$, $M_4 10^0$	16, 16
			2	U_3^5, U_3^6	$M_3 10^{-1.4}$, $M_4 10^{-1.4}$	13, 13

TABLE VIII.—*Analysis of Variance of the Data in Table VII.*

Source of variation.	D.f.	Mean square : Unknown preparations.
(A) Between rabbits	1	0
(B) Between days of growth	(2)	
Linear	1	1089***
Quadratic	1	1
(C) Between doses	1	656***
First order interactions		
(AB)	2	4
(AC)	1	26
(BC)	2	36
Error (a)	14	20·4
Error (b)	72	7·1
		Standard preparation.
(A) Between rabbits	1	39
(C) Between doses	(3)	
Linear	1	888***
Deviations	2	59**
(AC) interaction	3	0
Error (c)	24	2·6

** $0{\cdot}01 > P > 0{\cdot}001$; *** $P < 0{\cdot}001$.

diameter, corresponding with equal logarithmic increment of virus concentration. In the period of two days the virus is multiplying logarithmically.

Estimates of the potency of the six unknown preparations relative to the standard preparation may be made in the standard manner. These are unnecessary for the full interpretation of the data which is provided by the analysis of variance given above. Comparison of *error* (*b*) with *error* (*c*) by the F test ($P < 0{\cdot}001$) indicates that variation in response to the unknown preparations is considerably greater than variation in response to the standard preparation. While this would render invalid any simultaneous analysis of the data, it shows that the relatively greater impurity of the unknown preparations can increase variability of response.

DISCUSSION.

The dose response relationship between myxoma virus and lesion diameter is somewhat unusual in that it exhibits both graded and quantal (all or none) characteristics. The dose response curve may be arbitrarily divided into two segments, the point of division depending on the strength of a particular preparation. For doses above that of 10^{-4}–10^{-5} of our standard preparations lesion diameter is linearly related to log dose. This interval of linearity is very wide (4–5 units on the $\log_{10}$ scale) when compared with the region of linearity of some hormone assays (less than 2 units on the $\log_{10}$ scale). In this interval response behaves in a classical graded manner with respect to dose and biological assays may be designed accordingly (Emmens, 1948 ; Finney, 1952*a*).

Below this critical point the quantal nature of the response curve becomes dominant. Fenner and Woodroofe (1953) have given data on this interval from 10^{-5} to $10^{-7{\cdot}1}$ of virus. They found 100 per cent responses at a dose of 10^{-5} and 5 per cent responses at a dose of $10^{-7{\cdot}1}$. We have fitted a log dose probit response line (Finney, 1952*b*) to these data and it was found to have a slope of $2{\cdot}25 \pm 0{\cdot}21$ with $\chi^2_{(5)} = 5{\cdot}41$, $0{\cdot}5 > P > 0{\cdot}3$ which shows that Fenner's data are not discordant with the assumptions underlying the probit transformation of percentage response, *i.e.*, a log normal distribution of sensitivity of the rabbit skin to dose of virus. We have collected other data in this interval of dose and they agree fully with those of Fenner.

Thus if less than say 10^{-5} of virus is injected the skin has a certain measurable probability of not responding and if it does respond the lesion size is quite small. Averaging lesion size over those not responding and those responding will obviously give a dose response line falling rapidly below that predicted from the linear segment in the region where failure of response is highly improbable. The situation is illustrated in the Figure. For given precision assays in the quantal region require relatively many observations when compared to the number required in the graded section of the response curve. In our Latin square assays 100 observations gave an estimate of relative potency with fiducial limits of 67–149 per cent. With a probit slope of 2 units, 400 observations would be required for this precision (see for comparison the biological assays in Biggers and Claringbold, 1954).

The preliminary experiment showed that the killing time was also simply related to log dose. The slope of this log dose/log response line is very flat and again many observations are required for precision of the order of the percentage limits above. Thus about 550 responses, and hence 550 different rabbits, are required for the precision indicated, a clearly unpractical proposition. The great advantage

of lesion size as a measure of response is that many lesions may be obtained in a single animal.

It may be suggested that neighbouring lesions interfere, particularly when they are comparatively tightly packed over the skin of a test rabbit. Allocation to a square of the chess board of a particular unknown or standard preparation is therefore made completely at random and any such interference cannot bias the results, although it may contribute to error and reduce precision. The relatively high precision of the assay method suggests that this is unimportant even when up to 64 lesions are initiated on an individual rabbit. A quick method of randomisation may be mentioned for the chess board. The chess board squares may be numbered on rows or on columns; that is to say 1,8 stands for 1st row, 8th column.

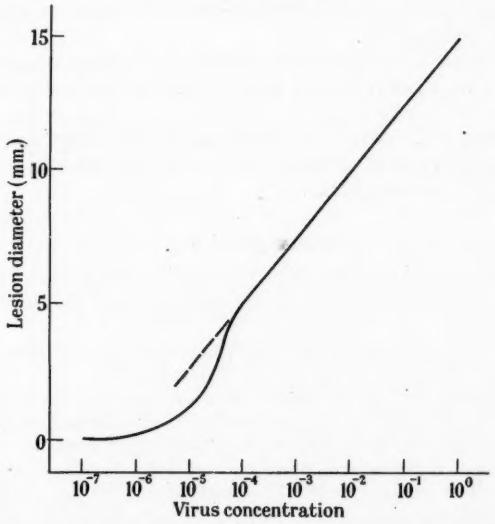

FIGURE.—The relationship of lesion diameter and log. dose. To the right of a virus concentration of 10^{-4} there is a linear dose response line. To the left of this concentration the response falls below that predicted on the basis of this linearity (see broken line) due to the quantal nature of the response in this region.

From Fisher and Yates (1953) Table XXXIII we may select a number at random between 11 and 88 ignoring zeros and nines and allocate a particular treatment to the square specified.

It has been shown that there is a logarithmic multiplication of virus in mouse brain for the first two days at least after inoculation. Therefore serial passage of myxoma virus through mouse brain should prove successful and may lead to the adaptation of the virus to the new tissue. Andrewes and Harisijades (1955) have recently carried out a more extensive investigation into the propagation of myxoma virus in mouse brain. They have confirmed the above suggestion in that 30 serial passages of virus in mouse brain have been made.

The method of assay introduced in this paper should be of use for assays of any virus which produces reasonably well demarcated skin lesions, such as fibroma

strains and vaccinia. It should be remarked that the assay must be used with great caution if different strains are to be compared since the lesions may differ qualitatively in form. The main advantage in the new method is that it enables rapid assay of relatively large amounts (100 ID_{50} units or more) of virus in terms of some standard.

SUMMARY.

Myxoma virus may be biologically assayed using lesion diameter resulting from intradermal injection of 0·1 ml. virus as a test criterion.

The lesion diameter is a more economical test criterion than either killing time or the quantal occurrence of lesions.

The characteristics of the dose response line are discussed with reference to the economical design of an assay.

In a practical test of the assay it has been shown that myxoma virus multiplies logarithmically for at least two days after intracerebral inoculation into mice.

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THE PORTAL BLOOD SUPPLY AND REGENERATION OF THE RAT LIVER.

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SINCE the early recognition of the rapid reconstitution of liver tissue following partial hepatectomy (Ponfick, 1890; 1895), many attempts have been made to elucidate the factors thought to control or stimulate this process. While there has been considerable difference of opinion in the assessment of experimental findings in the dietary, endocrine and biliary aspects of this problem (Cameron, 1935; Brues, Drury and Brues, 1936; Berman, Sylvester, Hay and Selye, 1947; Rapport, Canzanelli and Guild, 1948; Friedgood, Vars and Zerbe, 1950; Williams, 1951; Weinbren, 1953), there is almost unanimous agreement that the most important limiting factor in liver regeneration is the portal blood supply (Mann and Magath, 1922; Stephenson, 1932; Higgins, Mann and Priestley, 1932; Mann, 1940, 1944; Cameron, 1952; Child, Barr, Holswade and Harrison, 1953). Generally, the experiments planned to investigate the problem have consisted in comparing the mass of liver tissue after partial hepatectomy in animals with deficient portal blood flow with that in animals with unimpaired portal blood supply.

The conclusions reached have been similar in different animal species, and the dependence of liver regeneration on the portal blood flow has been accepted by most authors as established for the experimental animal and regarded as being probably true for human pathological processes (Himsworth, 1950; Child, 1954).

Appraisal of much of the experimental work suggests the possibility of inadequate control, since the changes induced in liver tissue by deprivation of the portal blood have not been distinguished from further changes induced by synchronous or subsequent partial hepatectomy (Williams). Anastomosis of portal vein and vena cava in the rat, the animal often used, is a very difficult and often ineffective procedure. The technique devised by Whitaker (1946), in which the two vessels are merely sutured together, is unreliable if complete deviation of portal blood from the liver is required. On the other hand, ligation and division of a branch of the portal vein, diverting portal blood from one lobe of the liver, is a relatively simple procedure. This is not complicated by the shock and infarction of the gut which usually follows ligation of the main portal vein, and it is therefore unnecessary to attempt porto-caval anastomosis. The lobe thus deprived of portal blood atrophies, while the other liver lobes with an intact portal blood supply undergo slight hypertrophy (Rous and Larimore, 1920). It seemed reasonable to attempt to apply this procedure to one of the lobes remaining after partial hepatectomy, and the degree of restoration might then be measured in liver tissue devoid of portal blood.

EXPERIMENTAL.

(1) Female albino rats weighing 190–290 g. were kept on a diet of " Research " rat cubes, litters being reared separately after weaning so that, so far as possible, experimental groups were accompanied by litter-mate controls. The animals were not starved pre-operatively or before death.

The following experiments were designed to compare changes in the right posterior lobe after partial hepatectomy in normal animals, with those in animals in which that lobe had been deprived of portal blood supply.

Group 1 : Partial hepatectomy in normal animals.

Removal of the anterior lobes (median and left lateral), approximately two-thirds of the total liver mass, was carried out under ether anaesthesia after the method of Higgins and Anderson (1931) in fifteen rats. Seven were killed after 3 days, and eight after 9 days, and the residual right and left posterior lobes weighed separately. In fifteen control rats, whose livers were removed under ether anaesthesia, the ratio of total liver weight to the weight of resected anterior lobes was 1·52 $\pm$ S.D. 0·034, and the right posterior lobe weighed 23·1 per cent $\pm$ S.D. 1·32 of the total liver weight (Fig. 3).

The factor 1·52 was used to calculate the total liver weight from the weight of the resected anterior lobes.

Group 2 : Ligation and division of the branch of the portal vein to the right posterior lobe.

This vein was separated from its accompanying arterial branch, and divided between two fine silk ligatures, in fifteen rats. Six were killed after 3 days, nine after 9 days, and each of the lobes was weighed separately.

Group 3 : Combined partial hepatectomy and division of the right posterior branch of the portal vein.

An operation combining the above two procedures was performed in fifteen rats by first dividing the portal vein branch and, immediately following, removing the two anterior lobes. Six rats were killed after 3 days and nine after 9 days, and each lobe was weighed separately.

Group 4 : Partial hepatectomy in the presence of established right posterior portal vein division.

In a series of thirty rats, division of the portal vein branch was carried out and, after 6 days, fourteen rats were killed and the liver lobes weighed separately. Partial hepatectomy and a small wedge biopsy of the right posterior lobe were performed on a further eight, and biopsy of the right posterior lobe, without partial hepatectomy, in a control group of eight rats. These last sixteen were killed 3 days later. This procedure was complicated by the fact that the liver lobes were always adherent to each other at the time of the second operation. Their separation, by incision of the capsule and blunt dissection, involved considerable trauma to the liver and a substantial degree of shock, so that it was considered necessary to separate the lobes in the control series in which only liver biopsy was taken at the second operation. This small fragment of liver tissue was immediately fixed, and sections were compared with those from the same lobe, when the animal was killed 3 days later.

The distribution of the portal blood flow was determined immediately before death under ether anaesthesia, in each case by injecting 0·2 ml. of 50 per cent India ink or 1 per cent methylene blue into the spleen, and the same quantity into the superior mesenteric vein (Himsworth, 1950), and by observing the staining of the liver lobes, both in their peripheral and hilar regions (Fig. 4) (Daniel and Prichard, 1951).

When the right posterior lobe was stained by this procedure, its portal vein having been divided, it was discarded. Livers with infarcts, following involvement of the hepatic arterial branch, were also discarded. After 10–20 sec. the main portal vein was clamped, the liver removed, the lobes were weighed separately, blocks were selected from each lobe for microscopical preparation, and samples were put aside for dry-weight determinations.

The numbers of rats in the four groups described refer to the total numbers finally recorded as successfully completed experiments, nineteen rats having died following the operation, and eighteen having been discarded because of infarction or staining of the test lobe, when dye was introduced into the extrahepatic portal circulation.

Dry weights were determined by drying in an oven at 105°–110° for a minimal period of 6 days, a constant weight having been achieved after 3 days, in order to check the possible part played by a change in the water content of the tissues.

Microscopical preparations were made on paraffin-embedded Helly-fixed tissue.

RESULTS.

The findings are summarised in Tables I and II, and in the graphs (Fig. 1 and 2). The weights of the right posterior lobe are expressed as percentages of total liver weights, as estimated from the anterior lobes, in all experiments involving partial hepatectomy (Brues *et al.*, 1936). In other experiments the weights of the right posterior lobe are expressed as percentages of actual total liver weights.

TABLE I.—*The Weight of the Right Posterior Lobe of the Liver after Partial Hepatectomy and Portal Deviation.* (Groups I, II, III.)

Number of rats.	Operative procedures.	Time in days.	$\frac{\text{Right posterior lobe}}{\text{Total liver}} \times 100.$ in %.	S.D.
15	— (normal control)	—	23·1	± 1·32
7	Partial hepatectomy.	3	44·7	± 3·74
8	„ „	9	63·8	± 7·91
6	Deviation of portal blood from right posterior lobe. Without hepatectomy.	3	10·6	± 1·96
9	Ditto	9	7·9	± 2·27
6	Partial hepatectomy + deviation of portal blood from right posterior lobe.	3	22·4	± 1·26
9	Ditto	9	22·0	± 2·80

TABLE II.—*Weight of Right Posterior Lobe 3 days after Partial Hepatectomy or Laparotomy in Cases of Established Portal Deviation.* (Group IV.)

Number of rats	Operative procedures.	Time in days.	$\frac{\text{Right posterior lobe}}{\text{Total liver}} \times 100.$ in %.	S.D.
14	I. Division of portal vein to right posterior lobe	6	7·3	± 1·46
8	I. Division of portal vein to right posterior lobe II. Partial hepatectomy 6 days later. Biopsy	3 (after Stage II)	22·9	± 3·90
8	I. Division of portal vein to right posterior lobe II. Laparotomy and biopsy 6 days later	3 (after Stage II)	6·7	± 1·43

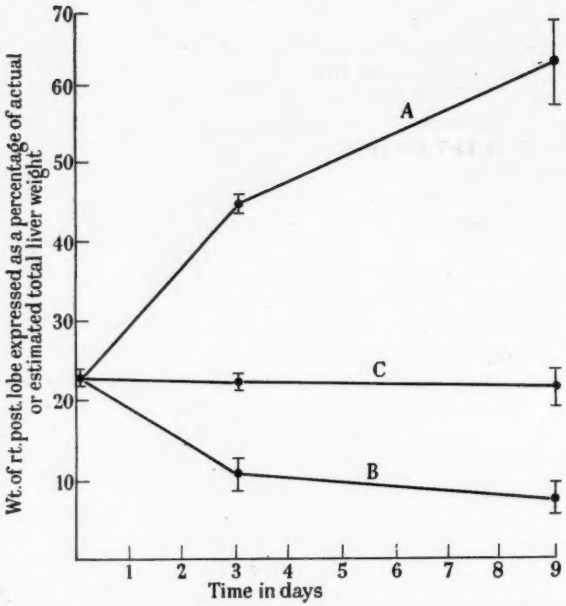


FIG. 1.—Graph showing alteration in weight of right posterior lobe after partial hepatectomy and portal vein branch division. Increase in moist weight of liver after partial hepatectomy and decrease after deviation of portal blood. The vertical lines represent + and − the standard deviation of the mean value. A = Right posterior lobe after partial hepatectomy. C = Right posterior lobe after partial hepatectomy and division of portal branch vein to right posterior lobe. B = Right posterior lobe after division of portal vein branch. No partial hepatectomy.

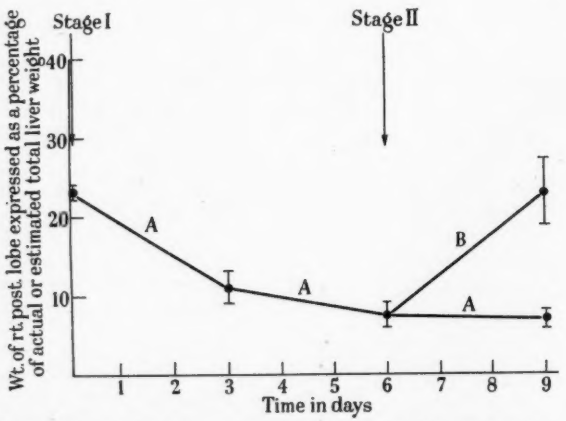


FIG. 2.—Graph showing decrease in moist weight of right posterior lobe after division of the portal vein branch (A) (Stage I operation), followed by an increase in the same lobe after partial hepatectomy on the 6th day (Stage II operation) (B). Right posterior lobe after division of portal vein branch (A). Same lobe 3 days after partial hepatectomy on 6th day (B).

Group I : Partial hepatectomy.

The mean weight of the residual lobes confirmed the findings of other workers, and also a large body of material from other experiments on the same strain of rats, the residual lobes doubling their weight in the first 3 days and, together, reaching almost 90 per cent of the estimated total liver after 9 days. The right posterior lobe reached 44·7 per cent (± S.D. 3·74) after 3 days, and 63·8 per cent (± S.D. 7·91) after 9 days (A in Fig. 1).

There was no significant difference in the water content of the residual lobes after 9 days. (Right posterior lobes 71·3 per cent, left posterior lobes 71·9 per cent.)

Group II : Division of portal vein to right posterior lobe.

The mean weight of the right posterior lobe was 10·6 per cent (± S.D. 1·96) of total liver in six rats, 3 days after operation, and 7·9 per cent (± S.D. 2·27) in nine rats, 9 days after operation (B in Fig. 1).

There was a slight increase in the water content of the right posterior lobes (71·7 per cent) as compared with the left posterior lobes (70·4 per cent).

Group III : Combined partial hepatectomy and division of the portal vein to the right posterior lobe.

The mean weight of the right posterior lobe was 22·4 per cent (± S.D. 1·26) of estimated total hepatic weight in six rats killed after 3 days, and 22·0 per cent (± S.D. 2·80) in nine rats killed after 9 days (C in Fig. 1).

The water content, after 9 days, in the right posterior lobes (72·0 per cent) was not significantly different from that in the left posterior lobes (72·4 per cent).

The right posterior lobe is thus significantly lighter when deprived of portal blood after partial hepatectomy than with an intact portal blood supply. It is, however, significantly heavier than the corresponding lobe deprived of portal blood in the otherwise intact liver (Fig. 5–8).

The weight of liver tissue after portal deviation and partial hepatectomy may therefore possibly represent the combined effect of two concurrent processes : atrophy of liver tissue following portal deprivation (Rous and Larimore) ; and regeneration of the lobe following loss of liver substance.

An alternative interpretation of the weight differences may be that the atrophy, which usually follows deprivation of portal blood, does not take place in the absence of intact anterior lobes, and that the weight changes therefore may not indicate regeneration.

The procedures involving vein division and subsequent partial hepatectomy were carried out in order to determine whether regeneration could be stimulated in liver tissue previously deprived of portal blood, and after considerable atrophy of the relevant lobe had already occurred. In this way it was hoped that the process of atrophy might be separated in time from the process of regeneration, if indeed these two components were responsible for the weight changes.

Group IV : Partial hepatectomy 6 days after division of vein.

In the fourteen rats killed 6 days after division of the branch of the portal vein, the ratio of total liver weight to the weight of anterior lobes was 1·32 (± S.D. 0·08), and the right posterior lobe weighed 7·3 per cent (± S.D. 1·46) of total liver weight. The water content of the right posterior lobe (72·9 per cent) was not

significantly greater than that of the left posterior lobe (72·2 per cent). The new factor, 1·32, was used for estimating total liver weight from the resected lobes in this group.

In the animals in this series subjected to partial hepatectomy, the right posterior lobe after 3 days weighed 22·9 per cent (± S.D. 3·90) of estimated total liver weight (B in Fig. 2). The corresponding lobe, in animals subjected to biopsy and separation of the liver lobes without resection, weighed 6·7 per cent (± S.D. 1·43) 3 days after the second operation (A in Fig. 2) (Fig. 9). There were no significant differences in water content between the right and left posterior lobes, the right posterior lobes containing 74 per cent, 3 days after either operation, the left posterior lobes containing 73·8 per cent and 73·3 per cent of water after partial hepatectomy and laparotomy respectively.

The findings in this group indicate that the lobe deprived of portal blood increased considerably in weight after partial hepatectomy. Microscopical examination showed an increase in cell and lobule size as compared with controls. There was also a high incidence of mitotic figures. In view of the diurnal periodicity of mitoses in regenerating liver (Jaffe, 1954), only rats killed between 10 a.m. and 4 p.m. were used for mitoses counts. The mitotic figures were counted in alternate high power fields (objective × 44, ocular × 10) after the method described by Jaffe. The mean incidence in the right posterior lobes of five rats subjected to partial hepatectomy 6 days after vein division, was 198 mitotic figures per 100

EXPLANATION OF PLATES.

FIG. 3.—Anterior and inferior view of normal liver of rat, showing relationships of liver lobes. The two anterior lobes have been turned upwards and the right posterior lobe is in the lower left corner of the photograph.

FIG. 4.—Posterior view of residual lobes of liver 2 days after partial hepatectomy and division of right posterior portal vein branch. The spleen and superior mesenteric veins were injected with India ink immediately before death and there is a distinct demarcation between the stained left posterior lobe and the unstained right posterior lobe.

FIG. 5.—Anterior and inferior views of livers from rats of comparable weights. (a) Normal liver (weight of right posterior lobe 1·9 g.). (b) Residual lobes 3 days after partial hepatectomy (weight of right posterior lobe 4·1 g.). (c) Total liver 3 days after division of portal vein branch to right posterior lobe, showing atrophy of right posterior lobe (weight 0·8 g.). (d) Residual lobes 3 days after partial hepatectomy and division of portal vein branch to right posterior lobe (weight of right posterior lobe 2·0 g.). The right posterior lobe of specimen (d) is much larger than the corresponding lobe in specimen (c) but equivalent in weight to that in specimen (a).

FIG. 6.—Section of normal right posterior lobe of liver; H. and E.; for comparison with Fig. 7. × 100.

FIG. 7.—Section of right posterior lobe 3 days after portal vein division; H. and E.; shows atrophy of liver cells. × 100.

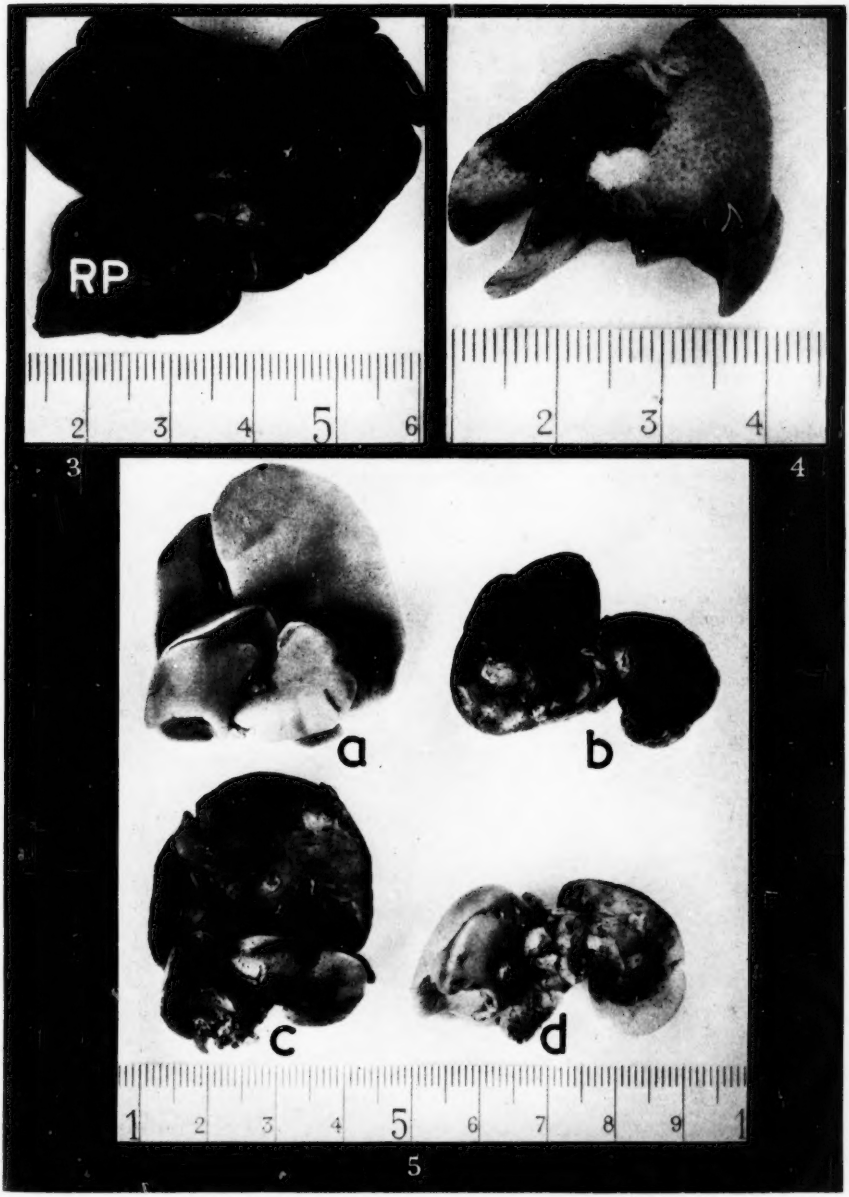
FIG. 8.—Section of right posterior lobe 3 days after partial hepatectomy and portal vein division; H. and E.; shows numerous mitotic figures. × 340.

FIG. 9.—Anterior and inferior views of: (on left) total liver, 6 days after portal vein branch division (Stage I operation) to right posterior lobe; (on right) residual liver lobes 3 days after Stage II operation (partial hepatectomy 6 days after portal vein division). In both specimens the right posterior lobes are unstained, whereas the remaining liver lobes are intensely stained by dye injected into the spleen and superior mesenteric veins. The right posterior lobe, deprived of portal blood, is much larger after partial hepatectomy than it is before this procedure.

FIG. 10.—Section of biopsy of right posterior lobe 6 days after portal vein ligation. H. and E.; shows atrophic liver cells. × 140.

FIG. 11.—Section of same lobe taken 3 days after partial hepatectomy from the same rat as Fig. 10. H. and E.; shows increase in size of liver cells and nuclei. × 140.

FIG. 12.—High power view of same section as FIG. 11. H. and E.; shows numerous mitotic figures in parenchymal cells. × 430.



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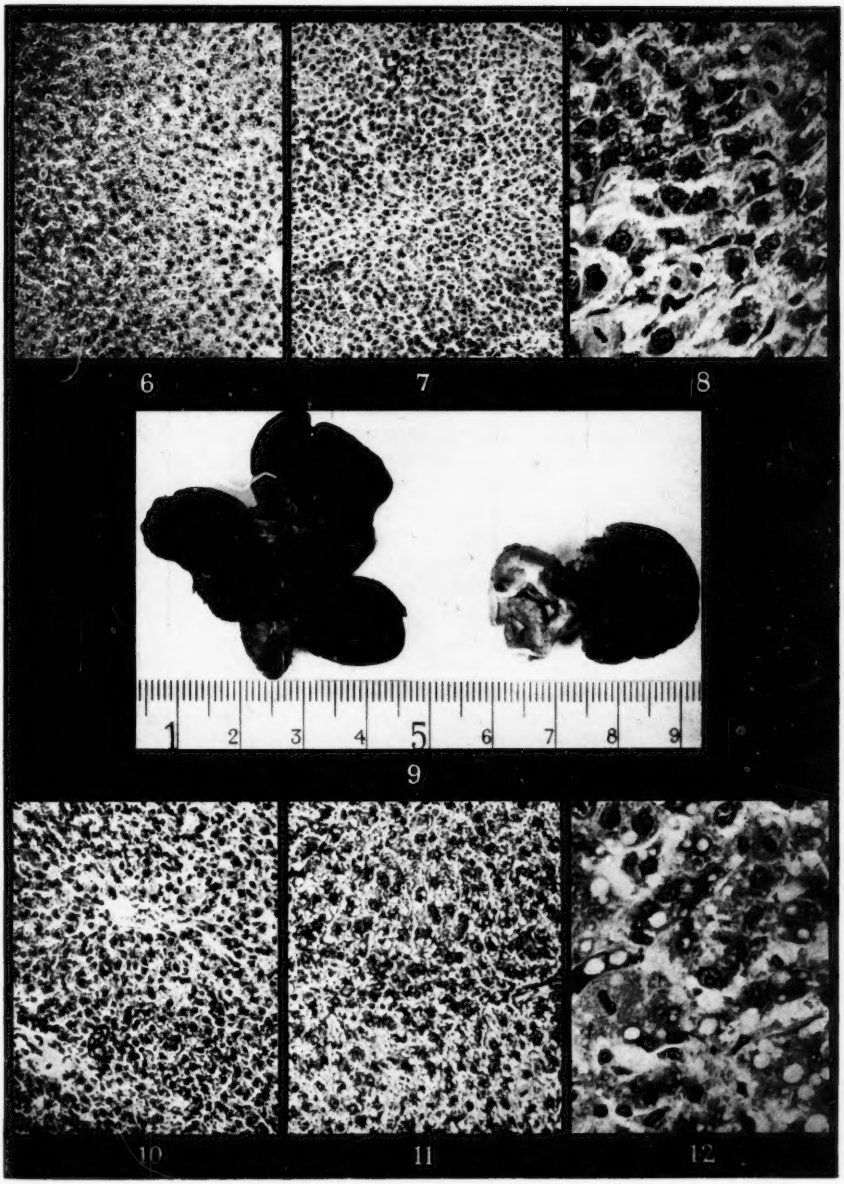
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high power fields (range 130–280), and no mitoses were found in the biopsy specimens or in the corresponding lobes taken from the control rats (Fig. 10–12). These microscopical findings are recognised as characteristic of regenerating liver tissue (Ponfick ; Brues *et al.*).

DISCUSSION.

The interpretation of these results was complicated by the problem of differentiating weight changes due to regeneration from those related to alterations in the portal blood flow. After vein division combined with partial hepatectomy, the weight of the test lobe was lower than that of the comparable lobe after hepatectomy only, and equivalent to that of the comparable lobe in a normal liver. This comparison, however, is not valid because in the latter two conditions the portal blood supply was intact and the final weights were not complicated by the changes induced by portal deprivation. The changes induced in liver tissue by the combined operation were therefore compared with those following vein division only, and it was shown that regeneration of liver tissue deprived of portal blood occurred after partial hepatectomy. Three days after resection the test lobe reached twice the weight of controls both with and without a portal blood supply and after 9 days approximately three times the weight of controls. After the combined operation the weights at these times were 22·4 per cent and 22·0 per cent respectively of the total liver weight, whereas the weights of similar lobes after deprivation of portal blood only were 10·6 per cent and 7·9 per cent respectively of the total liver weight. After partial hepatectomy with an intact portal blood supply the values were 44·7 per cent and 63·8 per cent respectively, compared with the normal control of 23·1 per cent. The extent of regeneration therefore, when the test lobe after the combined operation is compared with the relevant controls deprived of portal blood only, appears to be equivalent to that seen after partial hepatectomy with an intact portal blood supply.

In the work of others comparison has been made between liver tissue after simple partial hepatectomy and that after partial hepatectomy together with an operation restricting the portal blood flow. The conclusions reached after such experiments differ from those arrived at in the present series. It seems possible from the published work that the changes in liver tissue, induced solely by restriction of the portal blood supply, have not been distinguished from those following the combined operation of reducing the portal blood supply and resecting part of the liver.

Stephenson (1932) carried out partial hepatectomy and constriction of the portal vein in rats and found that at comparable times the residual liver tissue weighed less than residual liver after partial hepatectomy only. He concluded that the normal restoration of the organ was inhibited by the reduction of the portal blood flow. These experiments have been justifiably criticised by Williams (1951), who pointed out that adequate controls were not used to assess the effect of reduction of portal blood flow on the otherwise intact liver. It had been shown previously by Rous and Larimore that deviation of the portal stream from part of the rabbit's liver resulted in atrophy of that part. Higgins *et al.* (1932), in their study of liver restoration in the domestic fowl, found that after partial hepatectomy the residual liver tissue weighed more in cockerels when the systemic blood was shunted through the inferior mesenteric vein into the portal system. They concluded that liver regeneration was greater in those livers with a greater supply of

venous blood. The intact livers of cockerels also increased considerably in size after the portal blood supply was increased, but these livers were not used as controls for comparison with the partially hepatectomised series.

On the basis of experiments conducted on dogs, Mann (1940) postulated that regeneration after partial hepatectomy depends on the flow of portal blood through the liver. He suggested that " restoration of hepatic tissue occurs primarily in order to maintain the portal pathways and the restoration of functioning hepatic tissue is secondary " (1944). The experiments carried out by Mann were arranged so that comparisons were made between residual tissue following partial hepatectomy but with intact circulation, and residual tissue following partial hepatectomy and varying degrees of portal blood deprivation and porto-caval anastomosis. Controls indicating the effect of restriction of the portal blood supply on the intact liver were not used.

Grindlay and Bollman (1952) compared weights of residual lobes after partial hepatectomy in dogs with portal vein constriction, in dogs with reversed Eck fistulae and in dogs with portal and hepatic vein constriction. The weights of the residual lobes in the groups with portal and hepatic vein constriction were greater than those in the groups with portal vein constriction alone. They postulated that filling of the veins favours regeneration of the liver. In cases of regeneration following portal vein constriction and caval constriction below the ostia of the hepatic veins they suggested the possibility of stasis or reverse flow of blood in the inferior vena cava thus filling the hepatic veins. In these experiments only six of a total of sixteen dogs subjected to portal constriction and partial hepatectomy survived for longer than one week. Two of these had died, two were moribund when killed, and all were said to be in a very poor condition. Adequate controls, using the liver weights of dogs with portal constriction in a comparably poor condition, but without partial hepatectomy, were not used. It is well known that in starvation the liver weight is grossly reduced (Addis, Poo and Lew, 1936), and that a reduction also follows diminution of the portal blood flow. These two factors might be expected to cause a similar reduction in regenerating liver tissue, and in the absence of adequate controls such findings as those of Grindlay and Bollman do not prove a diminished regenerative capacity. The possibility of backflow of hepatic venous blood into the liver tissue deprived of portal blood is pertinent to the present investigation. In the experiments described the hepatic venous blood from liver lobes with portal blood supply mixed, in the inferior vena cava, with venous blood from liver lobes without portal blood supply. A reflux from the inferior vena cava might therefore contain blood derived from liver tissue with an intact portal blood supply. The dye injections performed immediately before death in all the experiments, were designed to exclude this possibility, and even a slight degree of staining of the test lobe was sufficient to invalidate that experiment.

Child *et al.* (1953) and Child (1954) carried out partial hepatectomy in eight dogs with transposed portal and caval blood flows, in six with normal hepatic circulation, and in one with Eck fistula. From a comparison of the weights of the residual lobes at the end of the experiment they concluded that regeneration depended largely on the volume of blood delivered to the liver, and to a lesser extent on whether it was caval or portal blood. The one dog with an Eck fistula was jaundiced and intermittently comatose, and died twenty-nine days after operation. At autopsy its right hepatic duct was tied and its liver was described as showing biliary

cirrhosis. In view of this complication the authors assumed from the work of others that liver regeneration in dogs with Eck fistulae " is zero ", and used this figure as a basis for comparison with their other experimental groups. Controls, in which the vascular operations were performed in the absence of partial hepatectomy, were apparently not used. In the absence of adequate controls the weight differences observed may reflect the result of changes in blood flow to the liver, and may bear little relation to the degree of restoration.

SUMMARY.

The changes after partial hepatectomy were observed in the right posterior lobe of the liver in normal rats and in rats in which the branch of the portal vein supplying that lobe had been divided.

Right posterior lobes in which the vein had been divided, with partial hepatectomy, showed a striking increase in weight, cellular and lobular size, and incidence of mitotic figures over right posterior lobes without portal blood supply in otherwise intact livers.

Deprivation of portal blood supply, whilst inducing considerable atrophy of liver tissue, does not impair its ability to regenerate after partial hepatectomy.

I wish to thank Professor Dorothy S. Russell for facilities and helpful criticism, Messrs. K. V. Swettenham and I. R. Holliday for the sections, and Mr. A. L. Gallup for the photographs.

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A METHOD FOR MEASURING THE RESPIRATION OF ANIMAL CELLS *IN VITRO*, WITH SOME OBSERVATIONS ON THE MACROPHAGES OF THE RABBIT.

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CONVENTIONAL manometric respirometers are unsuitable for measurements on living animal cells *in vitro* for two main reasons. First, most of these cells survive for only very short periods at 37° in the buffered salt solutions commonly used in manometric respirometry. If serum, or some other biological fluid in which the cells do survive, is used as the medium, the respiration of the cells can only be measured indirectly, and with considerable error. Second, in order to insure that the uptake of oxygen by the cells is not limited by its rate of diffusion through the medium, continuous agitation of the respirometer flask is necessary. Many cells are killed by this treatment. In the present paper a new method for measuring cellular respiration is described. The method is thought to have a general application in respirometry, but it has been more particularly designed for studies on living animal cells. Some observations carried out by this method on the macrophages of the rabbit are reported.

EXPERIMENTAL.

The respiration of the cells was measured by a modification of the electro-chemical circuit described by Tödt, Teske, Windisch, Heumann and Goslich (1952). Electrodes were introduced into a coverslip chamber which contained the cells. The chamber was completely filled with medium, so that there was no gas space. The oxygen uptake of the cells was determined by measuring the fall in the O_2 tension of the medium.

The cells.

Macrophages were obtained from the peritoneal cavity of the rabbit by the technique originally described by Lucké, Strumia, Mudd, McCutcheon and Mudd (1933). Paraffin oil was injected into the peritoneal cavity and the resulting exudate removed some days later. After separation from the paraffin oil, the cell suspension obtained was centrifuged at 220 *g.* and the cells re-suspended at a suitable density in a medium containing 25 per cent of rabbit serum in Hanks' solution (Hanks, 1948). This suspension was introduced into a coverslip chamber.

The chamber.

The coverslip chamber was a modification of the one devised by Mackaness (1952). A plan of the chamber is shown in Fig. 1. The fluid in the chamber was stirred by a steel wire enclosed in a fine glass tube which rested on the Perspex ring attached to the bottom coverslip. This wire was rotated magnetically. After the chamber had been filled with the cell suspension, it was incubated at 37°. The cells settled out on the bottom coverslip and became adherent to it in about 15 min. (Fig. 3). The medium was withdrawn after the macrophages had adhered

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to the bottom coverslip, in order to remove any other cell types. Lymphocytes do not adhere to glass and polymorphonuclear leucocytes adhere much less tenaciously than macrophages; the small number of these cells present in the exudate was thus removed by withdrawal of the medium.

The chamber was refilled completely with fresh medium. The O_2 tension and pH of this medium had been adjusted to the range required by gassing with either 5 per cent of CO_2 in oxygen, 5 per cent of CO_2 in air or 5 per cent of CO_2 in nitrogen. The number of cells adherent to the floor of the chamber was then counted directly under phase-contrast microscopy. The electrodes were introduced into the chamber through lateral drill-holes and the seals made air-tight with paraffin oil. The steel wire was rotated on the Perspex ring by placing the

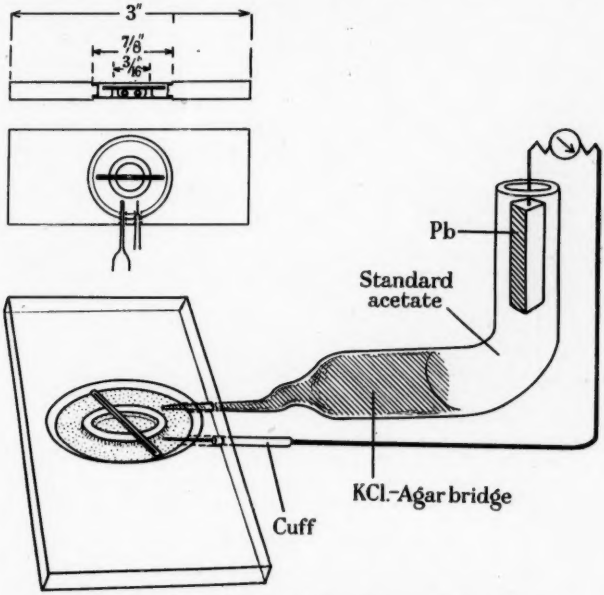


FIG. 1.—Plan of the electrodes and coverslip chamber.

chamber over a magnet which revolved at 30 to 40 r.p.m. This rate of stirring was found to give efficient mixing in the chamber, but it did not dislodge the cells from the bottom coverslip or injure them in any way. This was confirmed from time to time by direct microscopical observation. Since the stirrer itself was rotated on the top of the Perspex ring, it did not come into contact with the cells.

The volume of the chamber was determined gravimetrically for each experiment.

The electrodes.

The cathode consisted of platinum wire 0·018 in. (0·046 cm.) in diameter. This was coated with "Araldite"* resin except for a length of about $\frac{1}{8}$ in. (0·32 cm.) at one end. The uncoated end was immersed in the medium. A tapered polythene cuff was drawn over the resin-coated wire so that when the electrode was introduced into the chamber the cuff fitted tightly into the drill-hole. The anode was a piece of lead about 1 in. (2·54 cm.) long and $\frac{1}{8}$ in. (0·32 cm.) in diameter. This was immersed in standard acetate buffer contained in one end of a length of glass tubing about $\frac{3}{8}$ in. (0·95 cm.) in diameter. The other end of the tube was bent and drawn

* An adhesive resin supplied by Aero Research Ltd., Duxford, Cambridge.

out to a fine tip in which an isotonic KCl-agar bridge was incorporated. This tip could be inserted into the chamber through the second drill-hole (Fig. 1 and 2). Leads were soldered directly to the two metal electrodes and were connected to the terminals of a micro-ammeter.

Calibration of the electrodes.

When a noble metal cathode and a non-polarisable anode are immersed in an electrolyte solution and the circuit is completed, the current produced is proportional to the surface area of the cathode and the O_2 tension of the solution (Tödt *et al.*, 1952). Freier, Tödt and Wickert (1951) examined the relationship between the O_2 tension and the current produced in such a system and considered that it was not far from linear. In the above study the dissolved O_2 was measured by the toluidine-rhodamine method. Preliminary experiments with the electrodes described above led us to suspect that the relationship between O_2 tension and current was not linear, but logarithmic. A detailed calibration of the electrodes was therefore carried out, making use of Henry's law to vary the O_2 tension of the solution.

The electrodes were introduced into a 250-ml. vacuum flask containing some rabbit serum diluted 1/4 in Hanks' solution. The tips of the KCl-agar bridge and of the platinum cathode were immersed in the fluid. The leads from the electrodes were attached to brass screws which had been passed through the stopper of the vacuum flask. Leads were taken from these screws to the terminals of the micro-ammeter. The flask was connected through a mercury manometer to a suction pump, and the fluid in it agitated continuously by means of a magnetic stirrer. The calibration was carried out in an incubator at 37°.

To expel the gases initially dissolved in the medium the pressure in the vacuum flask was reduced until the solution boiled. Atmospheric air was then reintroduced into the flask, the fluid allowed to come into equilibrium with the gas phase, and a reading taken on the micro-ammeter. The pressure was then reduced in stages until zero was almost reached. After each change in pressure the fluid was allowed to come into equilibrium with the gas phase before the reading was taken. It was found that with continuous stirring equilibrium was attained under the present conditions in 15 to 20 min. ; the readings were therefore taken 30 min. after each change in pressure. The calibration was repeated using pure O_2 as the gas phase. The O_2 tension of the fluid was calculated from the level of the mercury manometer after a correction had been made for the pressure of water vapour in the flask.

The relationship between the current produced and the O_2 tension of the fluid is shown in Fig. 4. It will be seen that this relationship is not linear, approaching linearity only at high O_2 tensions. If, however, the current is plotted against the logarithm of the O_2 tension, a straight line relationship is obtained (Fig. 5). This relationship is given by the expression :

$$\text{Log } T = k_1 I + k_2$$

where T = the O_2 tension of the fluid, I = the current generated, and k_1 and k_2 are constants describing the characteristics of the platinum electrode. The values for these constants are obtained by empirical calibration of the electrode.

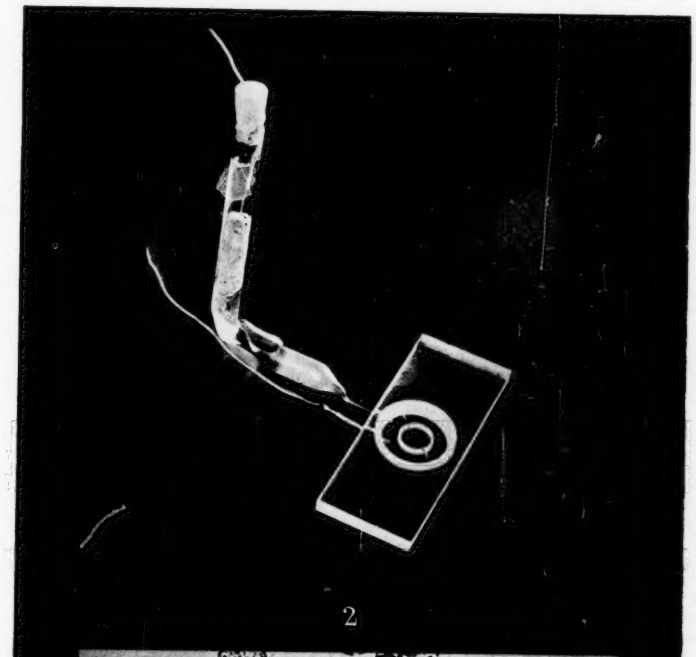
It will be noticed that a small current persists even when the pressure in the flask has been reduced virtually to zero. It is not known whether this current is due to the presence of traces of O_2 in the medium, or whether it is due to some intrinsic property of the electrode system. In any case, it is not possible to eliminate this residual current even if the medium is allowed to boil, or the O_2 in it is removed by chemical means.

Technique of taking the readings.

In order to avoid the continuous passage of current through the medium for many hours, the circuit was connected only while a reading was being taken. This made it necessary to standardise the technique of taking the readings. When the connection was made the initial reading was higher than the equilibrium level which could be correlated with the O_2 tension. Tödt (1950) has shown that for a given O_2 tension the height of this initial reading is a function of the time interval between readings. As the current flows, however, the reading rapidly falls

EXPLANATION OF PLATES.

FIG. 2.—The electrodes and chamber assembled.
FIG. 3.—Macrophages adherent to the floor of the chamber. (× 600).



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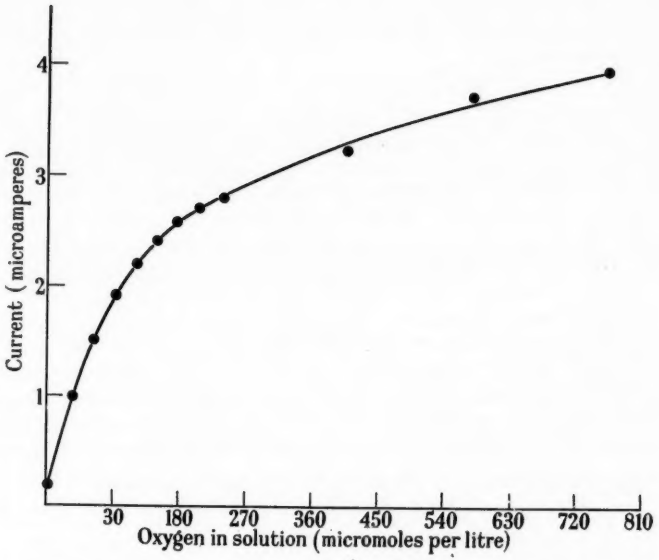

FIG. 4.—Calibration curve showing the relationship between O_2 tension and current.

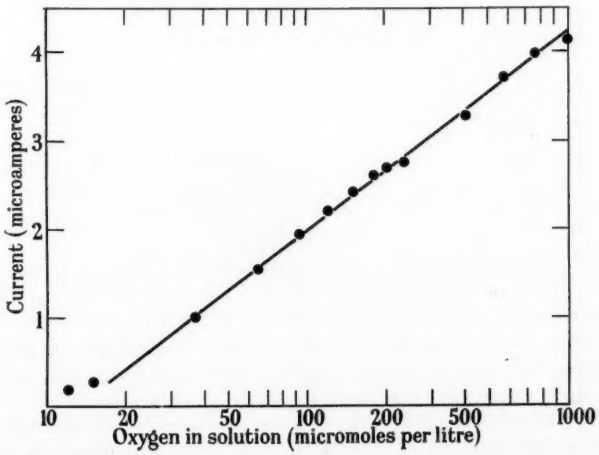

FIG. 5.—The relationship between the logarithm of the O_2 tension and the current.

to the equilibrium level. Under the present conditions, equilibrium was attained within a minute. All readings were therefore taken 1 min. after the connection had been made, and at intervals of exactly 30 min. With the technique of taking readings standardised in this way, there was no difficulty in obtaining an accurate and reproducible correlation between the current and the O_2 tension.

The effect of pH.

In order to determine whether the current produced was influenced by the changes in pH which might be expected to occur in the medium during cellular respiration, a series of Sørensen's buffers ranging from pH 5·9 to pH 8·1, and equilibrated with the same O_2 tension, were tested. Over this range the current was found to be independent of the pH.

Chemical methods.

The glucose concentration of the medium was determined by the method of Hagedorn and Jensen (1923); the lactic acid concentration by the method of Barker and Summerson (1941).

RESULTS.

The respiration of rabbit macrophages.

In Fig. 6 the results of a typical experiment are shown. The O_2 tension in the medium containing a given number of macrophages fell in a linear fashion until virtually all the O_2 was exhausted. The O_2 uptake of the cells was thus independent

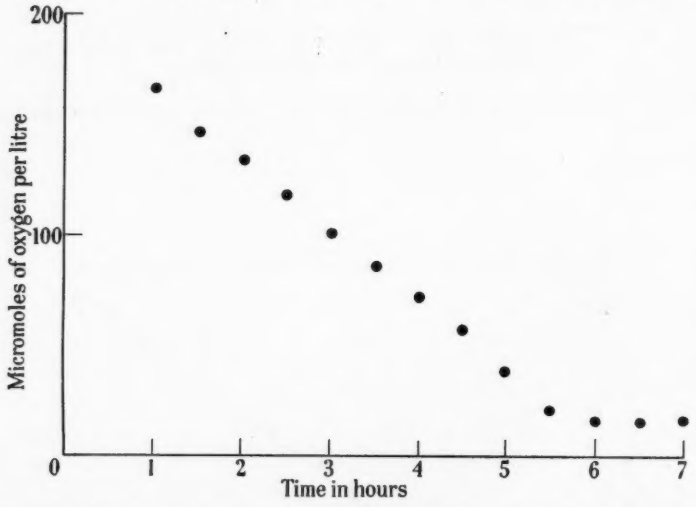

FIG. 6.—The fall in the O_2 tension of the medium in a chamber containing living macrophages. In this experiment the chamber contained 600,000 cells and 0·776 ml. of medium.

of the O_2 tension of the medium. It will be noted that there was a small residual current, as in the calibration curve (Fig. 5). If this current represents a trace of O_2 persisting in the medium, this O_2 is apparently unavailable to the cells.

The mean O_2 uptake of macrophages from 6 different exudates withdrawn from the peritoneal cavity on the fifth or sixth day after the injection of paraffin oil was 0·040 micromoles per 10^6 cells per hour (S.D. 0·006). Cells withdrawn from

the peritoneal cavity on the fourth day after injection had a somewhat higher O_2 uptake. The highest value recorded in an exudate withdrawn on the fourth day after injection was 0·091 micromoles per 10^6 cells per hour.

The behaviour of macrophages under anaerobic conditions.

The complete exhaustion of the available O_2 in the medium produced no observable effect on the behaviour of the macrophages. They remained motile and free from any visible degenerative change until the end products of their metabolism depressed the pH of the medium to levels below 6·8. It was not possible to distinguish microscopically between macrophages in an anaerobic environment and the same cells in the presence of O_2. Macrophages could be kept alive under anaerobic conditions for many days by periodic changes of medium. Before the fresh medium was introduced into the chamber, the O_2 in it was expelled by prolonged gassing with nitrogen, and the pH was adjusted with CO_2 and nitrogen. Medium treated in this way still contained a small amount of O_2, but this was consumed by the cells within an hour. Thus, in an experiment lasting several days, with daily changes of medium, the cells were in a completely anaerobic environment for at least 23 hr. out of 24.

The glucose consumption and lactic acid production of macrophages under aerobic and anaerobic conditions.

Since it was clear from the foregoing experiments that the macrophage was a facultative anaerobe, it was thought worth while to see whether a " Pasteur effect" could be demonstrated. The glucose consumption and lactic acid production of the cells under aerobic and anaerobic conditions were therefore compared. The results obtained from three representative experiments are given in the Table.

TABLE.—*Comparison of the Glucose Consumption and Lactic Acid Production of Macrophages under Aerobic and Anaerobic Conditions.*

	Age of exudate. Number of days after injection of paraffin oil.	Oxygen uptake.	Consumption of glucose.	Production of lactic acid.
		Micromoles per 10^6 cells per hour.		
Aerobic	4	0·090	0·17	0·33
Anaerobic		—	0·27	0·60
Aerobic	8	0·037	0·18	0·24
Anaerobic		—	0·27	0·58
Aerobic	4	0·091	0·20	0·44
Anaerobic		—	0·34	0·79

Under anaerobic conditions there was a marked increase in the amount of glucose consumed and the amount of lactic acid produced. This " Pasteur effect " could always be demonstrated, although its magnitude varied somewhat from exudate to exudate, as did the O_2 uptake and the consumption of glucose. In most experiments about twice as much lactic acid was produced under anaerobic conditions. Moreover, it will be noticed that even if all the O_2 taken up by the cells was used in the combustion of glucose, it would account for only a small part of the total amount of glucose consumed. It thus appears that even in the presence of O_2 most of the glucose metabolised is converted to lactic acid. Under anaerobic

conditions the energy requirement of the cells is apparently met by an increase in this glycolysis.

SUMMARY.

A new method for measuring cellular respiration is described. This method is particularly suitable for the study of animal cells *in vitro*, as measurement of respiration can be carried out under conditions which permit direct microscopical observation of the cells. The electro-chemical circuit used in this method is able, without amplification, to record changes in oxygen tension of 1–2 micromoles per litre. Some observations carried out with this technique on the macrophages of the rabbit show that these cells are facultative anaerobes. Under aerobic conditions they have a constant oxygen uptake which is independent of the oxygen tension of the medium. However, even in the presence of oxygen, most of the glucose consumed is converted to lactic acid. Under anaerobic conditions the energy requirement of the cells is met by increased glycolysis.

We are indebted to Mr. G. J. Wren for technical assistance.

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STUDIES ON THE PURIFICATION OF DIPHTHERIA TOXIN.

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THEORELL and Norlin (1937) who tried to purify diphtheria toxin, using organic solvents as precipitating agents, had most success with acetone at low temperatures. None of their preparations, however, was homogeneous at electrophoresis. In order to carry the purification a stage further they used the acid precipitation technique which had given good results in the hands of earlier workers (Glenny and Walpole, 1915; Locke and Main, 1928; Eaton, 1936; Boivin and Izard, 1937). It was a disappointment to find, contrary to the results obtained by others, that the preparations were too labile for acid treatment.

Diphtheria *toxoid*, however, tolerated more drastic treatment. Using precipitation with acid, followed by acetone or repeated fractionation with acid, Theorell and Norlin (1937) obtained preparations that were homogeneous at electrophoresis. The purity of these preparations was 1200 Lf units per mg. total N (Kjeldahl), which was surprisingly low, since Pappenheimer (1937) reported a purity of 2150 Lf units per mg. N for his purified diphtheria *toxin*. Pappenheimer's toxin was homogeneous at electrophoresis and ultracentrifugation (Pappenheimer, Lundgren and Williams, 1940; Pappenheimer and Petermann, 1941), and the value 2150 accorded with the best results obtained by Eaton (1936).

The Pappenheimer figure was regarded, for more than a decade, as representing the activity of pure diphtheria toxin (and toxoid). It was verified by many others, including the present author (Norlin, 1947*a*; Levene, Wyman and Edsall, 1949; Ross, 1949; Norlin, 1951). Recently, however, both European and American workers have obtained, with increasing frequency, higher activities, *i.e.*, 2500–3200 Lf units per mg. N, for their purified toxins and toxoids (Pope, Stevens, Caspary and Fenton, 1951; Lepow and Pillemer, 1952; Pope and Stevens, 1953). This would suggest that the figure of 2150 Lf units per mg. N does not represent the value for " pure toxin ".

Norlin (1951) stressed the lability of diphtheria toxoid and pointed out that purification of such substances involved not only elimination of foreign " impurities " but also the necessity of avoiding " denaturation " of the active principle. It is of great importance not to mix biologically active material with denaturation products, chemically more or less similar to the active substance, but without biological activity.

MATERIAL AND METHODS.

The toxin used in this work was obtained by growing *Corynebacterium diphtheriae* Park Williams no. 8 strain (obtained from the Wellcome Research Laboratories), in the casein acid-hydrolysate medium of Mueller and Miller (1941). We used a medium having a total N content of 0·2 per cent (w/v) and containing 1·5 per cent (w/v) maltose, but in all other

respects the original directions were strictly followed. The medium was placed in modified Roux bottles (Piorkorsky), giving a layer 1·2–1·3 cm. deep. After incubation at 35° for 7 days the bacilli were filtered off on ordinary filter paper, and the filtrate, after addition of 0·01 per cent merthiolate, sterilised by Seitz filtration.

In 1946–47 the toxin contained 30–60 Lf units per ml.; in 1948–49, 50–80 Lf units per ml.; in 1952–53 we regularly produced 80–110, but now we find no more than 60–70 Lf units per ml. At the same time as the concentration of the toxin in the culture filtrate was higher the amount in Lf units per mg. protein N rose to 2300–2600. These values are remarkably high, since the protein isolated by Pappenheimer (1937) and found to be homogeneous at electrophoresis and in the ultracentrifuge contained 2150 Lf units per mg. N.

For solubility analysis the toxin solution was concentrated by passage through ultrafilters prepared with 10 per cent collodion in acetic acid on Berkefeld candles, as had been done earlier by Wadsworth, Quigley and Sickles (1938). The solubility of the proteins in ultrafiltered diphtheria toxin was measured by Falconer and Taylor's technique (1946). A constant volume of toxin concentrate of pH 7 was added to 8–10 tubes. Saline and saturated neutral ammonium sulphate solution was then added to give a series with rising concentrations of ammonium sulphate (from 30 to 80 per cent saturation) in a constant total volume.

After 1 hr. the contents of the tubes were filtered, the filtrates dialysed and flocculation and protein N values determined. In the course of the investigation of such filtrates we often found losses in the flocculating activity which caused irregularities and even systematic errors in the solubility curves. From analyses of the precipitates, however, it was possible to obtain rapid and apparently reliable results, which could be used to compute the solubility of the biologically active substance in the filtrate. In this way experimental series 1 to 7 were performed.

In the last three series of experiments (no. 8–10) stronger concentrates were used, so that the toxin flocculation reaction could be carried out on the filtrates directly after dilution.

Regarding the protein nitrogen values, the same figures were obtained regardless of whether they were determined directly from the filtrates or estimated by taking the difference between the protein nitrogen of the original material and that of the precipitates.

The flocculation was measured by the method of Ramon (1922). A series with falling volumes of dilute diphtheria standard serum was prepared in 7 tubes. Then 0·5 ml. of toxin, properly diluted, was added to each tube. A flocculent precipitate first appeared in the tube where the toxin and the antitoxin were present in equivalent amounts. The maximal error of the method, as performed in this investigation, is $\pm$ 5 per cent. As a check, the strength of some preparations was confirmed by *in vivo* titrations by Jensen's intracutaneous rabbit method (1933).

Protein N was determined by precipitation with trichloracetic acid to 5 per cent (w/v) acid, washing with 2 per cent (w/v) acid, and Kjeldahl combustion. The micro-Kjeldahl procedure has an experimental error of less than $\pm$ 2 per cent. In this investigation, however, the protein N has to be determined in a strong solution of ammonium sulphate. Hence the error of precipitation with trichloracetic acid and washing was added to the error of the N analysis. Occasionally a 10 per cent difference was observed between duplicate determinations, but in a special investigation a standard error as low as $\pm$ 2·3 per cent was found.

EXPERIMENTAL.

Observations on the Lability of the Toxin.

Pappenheimer (1937) used evaporation *in vacuo* as a preliminary step in his purification method, and at first we used the same technique. If the diphtheria toxin was concentrated *in vacuo*, however, volatile bases including ammonia were given off and the alkalinity was lowered appreciably resulting in loss of flocculating properties and sometimes even in precipitation of protein. If, however, the initial material was made slightly alkaline (pH 8–8·5) before evaporation, no sign of "denaturation" was apparent. Yet even so, subsequent fractionations with ammonium sulphate, adsorption on aluminium hydroxide and elution gave purified preparations which generally showed prolonged flocculation times, and some could be titrated only by the blending technique (Glenny and Okell, 1924).

In our later experiments, therefore, ultrafiltration was invariably used instead of evaporation *in vacuo*.

Pappenheimer (1937) evidently did not find any deleterious effects from vacuum evaporation, but on the other hand he observed an analogous reaction when dialysing purified toxin, because he noted that dialysis had to be carried out against 0·1 per cent sodium bicarbonate.

The present author met with the same difficulty when trying to dialyse diphtheria toxin against distilled water. As the toxin solution gradually lost its buffering capacity during dialysis against water, the pH of the solution fell to values in the vicinity of 4·1, the isoelectric point of the toxin. During dialysis at a pH less than 5·5 the toxin was destroyed and the inactive product might even precipitate. Our comparatively pure labile toxins can be dialysed to equilibrium with 0·066 M phosphate buffer pH 6–8, and also with 0·02 M phosphate buffer pH 7, without any loss in activity.

A similar problem arises when diphtheria toxin is freeze-dried. To dry the ammonium sulphate fraction, the material must be dialysed against a dilute, slightly alkaline solution, as below.

Diphtheria toxin concentrate was precipitated by ammonium sulphate at 50 per cent saturation and neutral reaction. The ammonium sulphate concentration in the filtrate was then raised to 65 per cent saturation. One portion of the new precipitate was dissolved in a little water and freeze-dried directly. The powder was practically insoluble, and an extract of it (pH 4·55) had no flocculation activity. Another portion was dissolved, dialysed against a running 0·1 per cent solution of sodium bicarbonate and freeze-dried. In this case the substance was readily soluble and the flocculating activity was recovered to 100 per cent. The pH of the solution was 6·75.

Besides this " acid denaturation " there is another reaction, inconstant and causing only a limited loss of activity, which is shown in the following example.

The initial material was an ultrafiltered toxin concentrate. Preparation was completed on March 19, 1953, and it was used for this experiment on May 11, 1953. It contained 720 Lf units per ml., and had MRD–$1.5.10^{-7}$ (*ca.* 10000 MRD/Lf). The pH was adjusted with 40 per cent NaOH to 8·0–8·5; then 158·5 g. solid ammonium sulphate were gradually added to 500 ml., the reaction never becoming less alkaline than pH 7·10. The precipitate was discarded and the mother liquid precipitated in the same way as before with 109·5 g. solid ammonium sulphate at pH 7·0 or slightly higher. The final precipitate was dissolved in water to a volume of 50 ml.

This solution contained 7000 Lf per ml. when flocculated the day after it had been prepared. The protein N was 0·215 per cent (w/v), giving an activity of 3260 Lf units per mg. protein N. The yield of the active principle was 97 per cent. This high yield means that no toxin had been included in the first precipitate obtained by 50 per cent saturation with ammonium sulphate at neutral reaction. This is unusual even with our ultrafiltered concentrates and, in our solubility investigations, to be mentioned later, has appeared only 3 times in 12 experiments. Two days later, after having been stored at + 4°, the preparation contained, however, only 5000 Lf units per ml., and in that short time had lost almost 30 per cent of its activity. Phosphate buffers of different pH values, sodium chloride, Ringer's solution and glycine buffers did not stabilise these highly purified toxin preparations.

This loss in activity amounting to 10–30 per cent has been observed a dozen times in ammonium sulphate fractions from batch U.55 during the first few months after its preparation. All efforts to inhibit the process, which ceases after 2–3 days storage at 4°, have failed. It is of interest, however, that the activity of these preparations initially exceeded 3000 Lf units per mg. protein N. After the activity had diminished in the way just described, it remained constant at the usual level for this laboratory, *i.e.*, 2600–3000 Lf units per mg. N.

Solubility Analysis.

Pappenheimer and Petermann (1941) showed that preparations 30 per cent less active than those isolated by Pope *et al.* (1951) were homogeneous at electrophoresis, diffusion and sedimentation. Preparations as active as those of Pope *et al.* have been obtained in this laboratory. It was therefore believed that a solubility analysis by Falconer and Taylor's (1946) method would reveal differences in biological activity even in physically homogeneous material.

Fig. 1 and 2 record the results of three experimental series. The curves start with a horizontal part, indicating that all flocculating activity is present in the

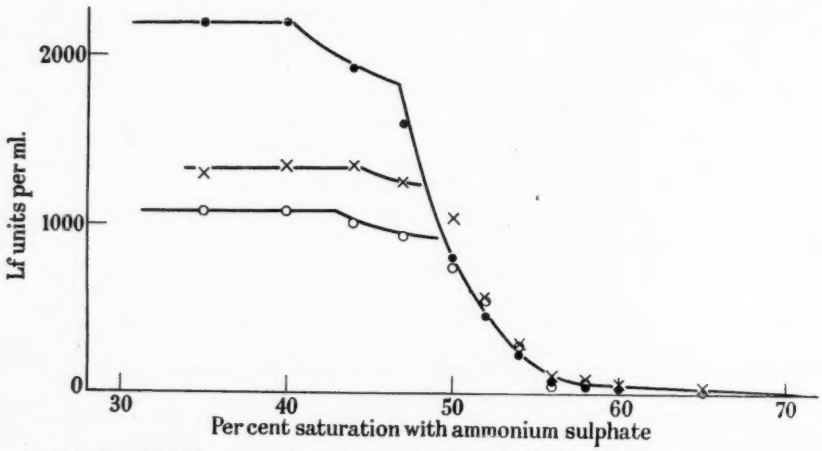

FIG. 1.—Solubility curves for flocculating principle in diphtheria toxin concentrates. Series 8 ● —— ● ; series 9 ○ —— ○ ; and series 10 × —— ×.

filtrate. Then two exponentially falling steps show that two flocculating principles, one less and one more soluble, are precipitated. Since the initial concentration of the toxin was not equal in the three series, the curves start on different levels. From the point where each solution is saturated with both components the three curves coincide.

Fig. 2 shows that there are two main proteins, one precipitated between 40 and 49 per cent saturation with ammonium sulphate, and the other between 49 and 60 per cent saturation. Since the curve for protein solubility does not reach the abscissa at 60 per cent saturation, as in the activity curve of Fig. 1, there must exist a small fraction of inactive protein of high solubility.

In Fig. 3, the flocculating activity from Fig. 1 is plotted against protein N from Fig. 2 to give a clearer picture of the protein fractions in series 8–10. The curves start from the origin with a straight horizontal part coinciding with the abscissa (series 9) or a curve (series 8 and 10). In each this part corresponds to the region of high ammonium sulphate concentration where an inactive protein, more or less free from diphtheria toxin, is left in solution. The inclined straight line shows that a toxin protein of constant activity can be separated from impurities. The last

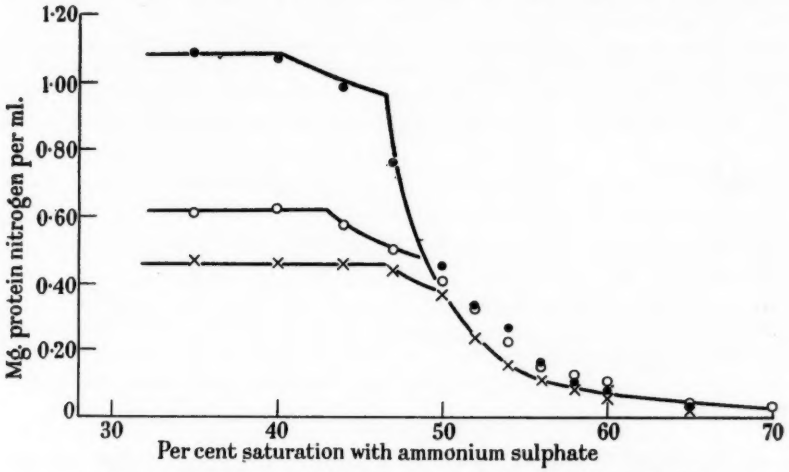

FIG. 2.—Solubility curves for protein in diphtheria toxin concentrates. Series 8 ●——●; series 9 ○——○ and series 10 ×——×.

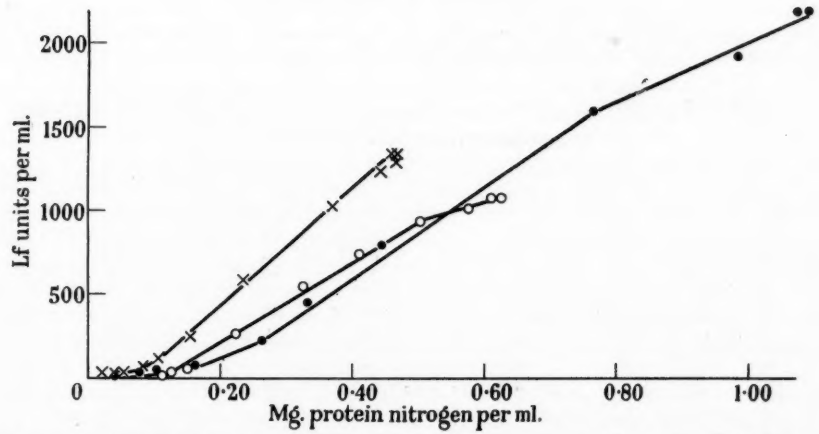

FIG. 3.—Relationship between flocculating activity and protein N in filtrates from solubility experiments of diphtheria toxin concentrates. Series 8 ●——●; series 9 ♀——♀ and series 10 ×——×.

part coincides with the least soluble protein in the initial material, which is serologically active and apparently easily separable from the toxin, since no overlapping zone seems to exist.

In other cases, of which Fig. 4 is an example, the last parts of the curves are horizontal, indicating that the least soluble protein is without flocculating activity.

From the curves of Fig. 3 and 4 and from similar curves of five other experiments, the three fractions of ultrafiltration concentrates were computed (Table I). Some variation between different batches is likely, especially regarding the more soluble protein fraction.

Series 10 needs special comment. It was carried out with a concentrate from the toxin that had been investigated directly after it was prepared as series 8. The concentrate was then stored in the cold room for 8 months, during which time the activity was unchanged. A dark precipitate settled, and after filtration the clear

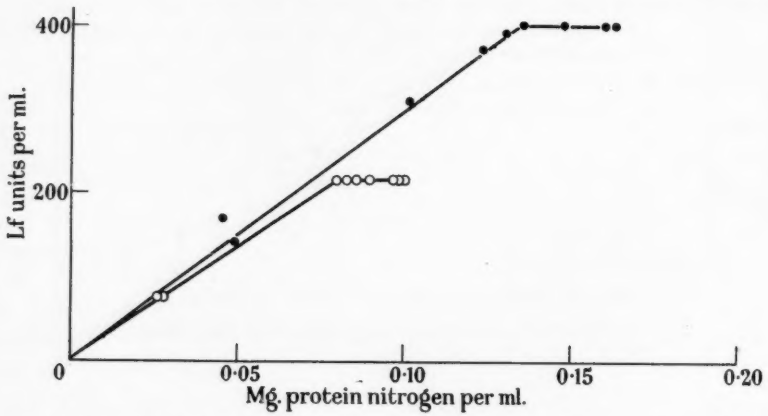

FIG. 4.—Relationship between flocculating activity and protein N in filtrates from solubility experiments of diphtheria toxin concentrates. Series 3 o —— o and series 4 ● —— ●.

TABLE I.—*Diphtheria Protein Fractions Obtained from Diagrams of the Activity/Protein N Relation.*

Series No.	Less soluble fraction. mg. PN/ml.	Less soluble fraction. Per cent.	Toxic fraction. mg. PN/ml.	Toxic fraction. Per cent.	More soluble fraction. mg. PN/ml.	More soluble fraction. Per cent.
1	0·062	28	0·147	66	0·014	6
2	0·067	30	0·155	70	0	0
3	0·020	20	0·080	80	0	0
4	0·033	15	0·131	80	0	0
5	0·026	14	0·150	85	0	0
6	0·008	14	0·047	81	0·003	5
7	0·007	13	0·048	87	0	0
8	0·325	30	0·580	53	0·185	17
9	0·125	20	0·390	62	0·110	18
10	Trace	Trace	0·390	84	0·074	16

In the Diagrams—of which Fig. 3 and Fig. 4 are examples—the different fractions are mostly represented by straight lines. The protein N figures can in these cases be directly read on the abscissa. In some cases the lines approach the abscissa as a curve. In those cases the straight line sextended until it reaches the axis and the point of crossing used in the analysis.

filtrate was diluted and used for solubility analysis. The precipitate contained most of the less soluble fraction, as only a trace of it was found on analysis.

The activity (Lf units per mg. N) of the toxin can be computed from the slope of the lines in Fig. 3 and 4. The results of the ten experimental series are shown in Table II.

TABLE II.—*Lf Units per mg. Protein N of Diphtheria Toxin.*

Series No.	Number of points on straight line.	Lf units per mg. protein N.
1	5	3240±120
2	3	3060
3	3	2690
4	6	3060± 60
5	5	3750±130
6	4	2900
7	4	2800
8	4	2770
9	5	2420± 40
10	6	3300± 60

The results provide no definite answer to the question whether the flocculating activity per mg. protein N is a constant or varies from one preparation to the other. The figure of 3000 Lf units per mg. protein N seems, however, to represent approximately the activity of a substance with constant solubility. This relative activity is consistent with the findings of Pope and his colleagues (1951 ; 1953) and with the preliminary results in this laboratory.

DISCUSSION.

Norlin (1947*b*) found that in a casein hydrolysate medium the toxin constituted 35–50 per cent of the total soluble protein synthesised by *C. diphtheriae* in seven days, the numerator being deduced by multiplying the number of flocculation units by 0·00046 (Pappenheimer, 1937) to get milligrams of toxin N. Judging from recent work (Pope and Stevens, 1953), the coefficient 0·00033 is more accurate. If this coefficient is used, the relative amount of specific toxin N in the culture filtrates from 1947 will be reduced to 25–35 per cent. Now, seven years later, the production of toxin has improved both quantitatively and qualitatively, for 60–80 Lf units per ml., constituting about 75 per cent of the protein, is produced. As before, about 0·2 mg. total soluble protein per ml. is synthesised by the bacilli.

The history of the flocculating activity of diphtheria toxin is interesting. The value reported by Pappenheimer (1937), 2150 Lf units per mg. N, was regarded for more than a decade as the activity of "pure" toxin. The physical analyses of Pappenheimer (1937) and Pappenheimer *et al.*, (1940) exclude the possibility of any major error in this figure. Nevertheless, we often find to-day 3000 Lf units per mg. N. Even though it is conceivable that quite a different protein has been synthesised in recent years, it seems far more likely that earlier preparations were more "contaminated" with biologically inactive protein—physically indistinguishable from "native" toxin—than is the case to-day.

It is of interest that Marhovich and Vorob'ev (1952) made an analysis of tetanus toxin very similar to that used in this investigation. They found that the activity, as estimated from their diagrams, closely accorded with that of the crystalline tetanus toxin (3520 Lf units per mg. protein N). There was one more soluble and one less soluble modification of the tetanus toxin,

The inactivation of ammonium sulphate fractions of some toxins is interesting. It starts relatively rapidly, so that 10–30 per cent of the flocculating activity is lost in a few days. The remaining toxin then is stable, and can be stored for several months in the cold room without appreciable loss of activity. Solubility analysis has shown that there is one protein of lower solubility which, in some batches, has a flocculating activity less than 2000 Lf units per mg. protein N, in others no activity at all. As this fraction constituted 10–30 per cent of the total protein, the quantitative data make it likely that this part is a very labile antigen, the flocculating activity of which is lost in most preparations.

SUMMARY.

Diphtheria toxin is a labile substance. Ultrafiltration is therefore preferable to vacuum evaporation, and dialysis and freeze-drying need special care, to avoid acidification and loss of flocculating activity.

Solubility analyses of diphtheria toxin concentrates point strongly to the existence of a homogeneous toxic protein, readily separable from less active material. Some batches showed variations in the activity of this toxin, but our preparations mostly contained about 3000 Lf units per mg. protein N. This accords with recent findings in the literature. Besides this toxin there is an extremely labile, less soluble protein, the flocculating activity of which was rapidly lost.

The difference in specific activity between older preparations and more recent ones is discussed. The divergence probably depends on the lability of the toxin. This lability makes it impossible—at present—to obtain preparations with more than about 3000 Lf units per mg. protein N.

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PROPAGATION OF FOWL PLAGUE AND OF NEWCASTLE DISEASE VIRUSES IN CULTURES OF EMBRYONIC HUMAN LUNG.

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WITH the object of finding a suitable virus to use for interference experiments in cultures of human tissues, attempts were made to propagate the agents of fowl plague and of Newcastle disease in roller tube cultures of embryonic human lung. Both these viruses are naturally pathogenic for birds. Newcastle disease virus has been reported to cause disease in man (Burnet, 1943 ; Placidi, Santucci and Haag, 1952). To our knowledge no case of human infection with fowl plague virus has been reported.

In the present paper we describe the successful propagation of each of these viruses in roller tube cultures of embryonic human lung and the development of a variant of fowl plague virus differing from the parent strain in its capacity to cause cytopathic effect in these cultures.

MATERIALS AND METHODS.

The viruses used were the Herts strain of Newcastle disease and the Dutch strain of fowl plague.

Cultures of human lung tissue were prepared from embryos up to 16 weeks of age. Roller tube cultures were prepared with 5 explants embedded in chicken plasma clotted by chick embryo extract. In some experiments the explants were placed on plasma-coated coverslips inserted in roller tubes. The nutrient medium (Enders, 1953) consisted of 90 per cent bovine amniotic fluid, 5 per cent inactivated horse serum and 5 per cent bovine embryo extract with the addition of penicillin (100 u./ml.), streptomycin (0·1 mg./ml.), soya bean trypsin inhibitor (0·1 mg./ml.) and phenol red (0·01 mg./ml.). The cultures were incubated at 37° in a revolving drum and the nutrient fluid was changed every 3 or 4 days. To avoid the accidental introduction of fowl cells into the cultures, the plasma and chick embryo extract used for the clot were filtered through a 0·7μ gradocol membrane.

After a few days' incubation a fairly extensive outgrowth, consisting almost exclusively of fibroblasts, could be observed around each explant. Cultures of varying ages were used for the experiments. Virus was added in appropriate dilutions to the medium. Passages were made into fresh cultures by the inoculation of the fluid medium from infected cultures in dilutions of 10^{-1} to 10^{-3}. Coverslips were stained by May-Grünwald-Giemsa according to the method described by Jacobson and Webb (1952).

Virus multiplication was estimated by egg infectivity and haemagglutination tests of the culture fluids. Egg infectivity titrations were carried out by allantoic inoculation of 0·05 ml. of tenfold dilutions of the virus preparations into groups of 4 ten-day embryonated eggs. The end points were calculated by the method of Reed and Muench (1938) and the results given as the number of 50 per cent infectivity doses (EID_{50}) per ml. Haemagglutination tests were performed by the pattern technique using 0·2 ml. of twofold dilutions of the culture material and an equal volume of a 0·5 per cent suspension of chicken red cells.

RESULTS.

Newcastle disease virus was propagated in cultures of embryonic human lung through a series of 8 passages (see Table I). The original inoculum was diluted by

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these passages to 10^{-14} not including the dilution due to fluid changes. A virus titre of $10^{5\cdot3}$ EID_{50}/ml. was obtained in the final passage. No cytopathic effect was observed throughout these passages and haemagglutinating titres were not reached.

TABLE I.—*Passages of Newcastle Disease Virus in Cultures of Embryonic Human Lung.*

Passage.	Inoculum.	Log EID_{50} in inoculum.	Log EID_{50}/ml. in successive fluids. F1.	F2.	F3.	F4.	F5.	Dilution of original allantoic fluid.
1	Allantoic fluid	7·1	—	5·5	3·9	4·3*	4·5	10^{-3}
2	P1–F4	2·3	4·9	5·1*	3·9	4·3	—	10^{-5}
3	P2–F2	3·1	—	—	—	—	>5·0*	10^{-7}
4	P3–F5	>4·0	—	—	3·7*	—	—	10^{-8}
5	P4–F3	2·7	—	3·7*	4·5	—	—	10^{-9}
6	P5–F2	2·7	3·2*	—	—	—	—	10^{-10}
7	P6–F1	2·2	2·8	4·9*	—	—	—	10^{-11}
8	P7–F2	1·9	5·3	4·7	2·3	—	—	10^{-14}

P1, P2....: First, second....passages.
F1, F2....: First, second....fluids removed from cultures.
* Material used as inoculum for next passage.

TABLE II.—*Passages of Fowl Plague Virus in Cultures of Embryonic Human Lung.*

Passage.	Inoculum.	Log EID_{50} in inoculum.	Log EID_{50}/ml. in successive fluids. F1.	F2.	F3.	F4.	F5.	Dilution of original allantoic fluid.
1	Allantoic fluid	6·7	—	—	7·3	6·5*	6·8	10^{-3}
2	P1–F4	4·5	>3·0	4·9*	6·7	4·1	—	10^{-5}
3	P2–F2	3·9	—	4·4*	—	—	—	10^{-6}
4	P3–F2	3·4	—	5·7*	>6·0	—	—	10^{-7}
5	P4–F2	4·7	>7·0*	—	—	—	—	10^{-8}
6	P5–F1	>4·0	4·9*	—	—	—	—	10^{-11}
7	P6–F1	3·9	5·5	—*	—	—	—	10^{-12}
8	P7–F2	—	7·5	—	—	—	—	10^{-14}

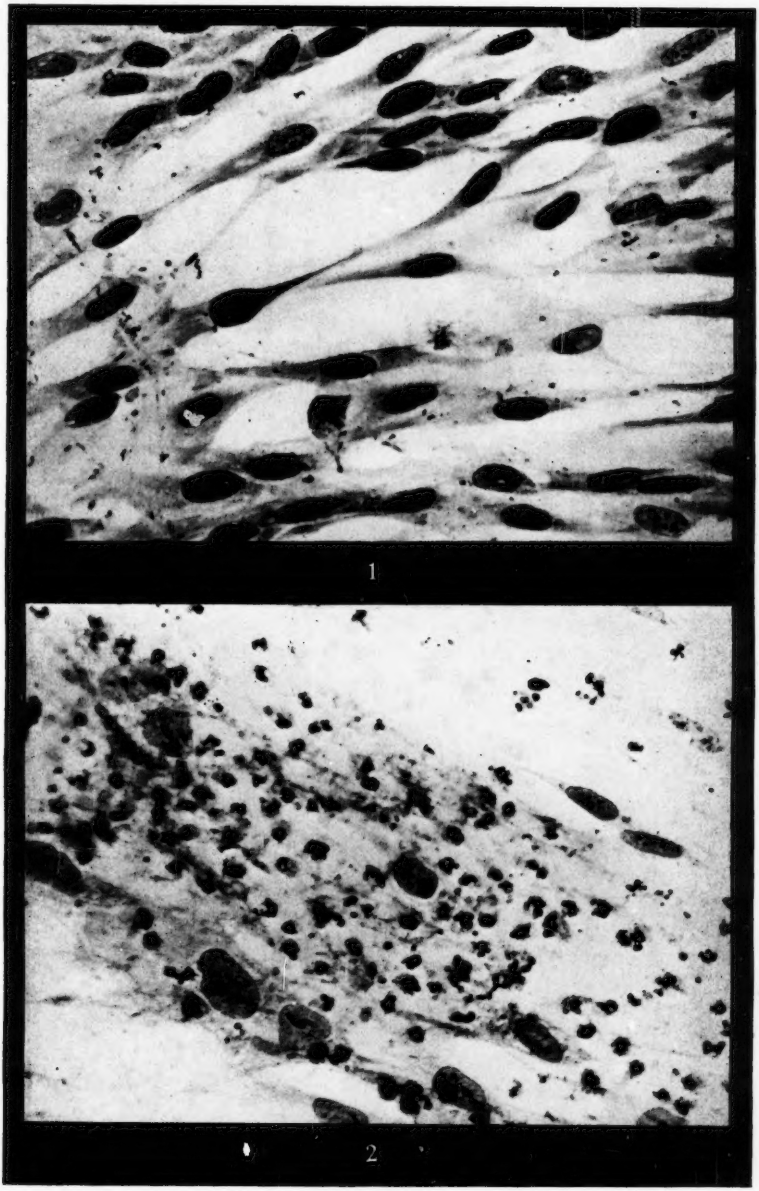
P1, P2....: First, second....passages.
F1, F2....: First, second....fluids removed from cultures.
* Material used as inoculum for next passage.

Fowl plague virus also was propagated through 8 passages in cultures of embryonic human lung (see Table II), the original inoculum having been similarly diluted to 10^{-14}. A virus titre of $10^{7\cdot5}$ EID_{50}/ml. was reached at the final passage. One of 2 experiments at the third passage and 2 of 5 experiments at the seventh passage failed to reveal virus multiplication. No explanation could be found for these failures, which were not related to the size of the inoculum, the origin of the tissues or to the age or microscopical appearance of the cultures at the time of inoculation. Haemagglutination titres were reached very irregularly throughout

EXPLANATION OF PLATE.

FIG. 1.—Uninoculated culture of embryonic human lung. May-Grünwald-Giemsa. × 700.

FIG. 2.—Culture of embryonic human lung 8 days after inoculation with human-adapted (sixth passage) fowl plague virus. May-Grünwald-Giemsa. × 700.



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the series, but developed earlier and occurred more frequently at higher passage levels. No cytopathic effect was observed in the first two passages. At the third passage this effect was observed in 1 of 2 successful experiments after 7 days' incubation. In further passages the cytopathic effect occurred with more regularity and its time of onset was reduced to 2 to 3 days. In two experiments in the seventh passage in which there was no cytopathic effect, virus multiplication did not occur.

The cytopathic effect caused by fowl plague virus is shown in Fig. 1 and 2. The first evidence was the appearance of basophilic granules in the nucleus. As degeneration progressed the nucleus became increasingly basophilic until the nucleolus and granules were no longer visible. At this stage the nucleus, often lobed or dumb-bell shaped, came to occupy one side of the cell and had by then become pyknotic. These changes were followed by complete cellular disintegration. Not all the cells were destroyed by the virus. The surviving cells continued to multiply, and after prolonged incubation the proportion of degenerating cells decreased until finally only débris and healthy cells could be seen.

Multiplication of fowl plague virus in cultures of human lung could be demonstrated in all passages by egg infectivity tests. The cytopathic effect in human lung cultures, however, was only observed from the third passage onwards and it occurred in cultures inoculated with lower doses than that used in the first passage. This result was interpreted as being due to the selection of a human-adapted variant of the virus. Material from the eighth human lung passage of fowl plague virus after three limit-dilution passages in embryonated eggs showed no change in its acquired cytopathic activity for human lung cultures. The same material was titrated in eggs and in human lung cultures, giving titres of $10^{-7.5}$ and $10^{-5.8}$ respectively. These end-points were calculated on the basis of haemagglutination tests in the egg titration and of cytopathic effect in the human lung cultures.

The human-adapted strain of fowl plague virus was compared with the parent strain for cytopathic effect in cultures of chick embryo fibroblasts and no differences could be detected.

Fowl plague and Newcastle disease viruses after 8 passages in human lung were each tested by haemagglutination-inhibition against immune sera and no differences from the parent strains were detected.

COMMENTS.

Evidence for the multiplication of fowl plague and of Newcastle disease viruses in the system under study was afforded by the frequent increase of virus titres in the cultures and by the demonstration of viral multiplication at the end of each series of passages, where the original inocula had been diluted to 10^{-14}. In the course of these passages no change of properties of Newcastle disease virus could be detected, but a variant of fowl plague virus, differing from the parent strain in its cytopathic effect in human lung cultures, was developed. This variant did not revert to the parent form after three passages in embryonated eggs at limit dilutions. A feature of most cultures showing cytopathic effect of fowl plague virus was the survival of some of the cells which continued to multiply and remain healthy after relatively long periods of incubation. This phenomenon may have been due to variations in the susceptibility of different cells or to interference, either by non-infectious virus retained in the solid phase of the culture

(Pereira and Gompels, 1954) or by the original non-cytopathogenic virus which might have accompanied the variant through the series of passages. Interference with poliomyelitis virus by a non-cytopathogenic variant was observed by Sabin (1954) and similar findings have been reported by Le Bouvier (1954). One experiment designed to demonstrate interference between the original and the human-adapted strains of fowl plague gave a negative result.

Recent work in our laboratory (unpublished) revealed that neither the parent nor the human-adapted strain of fowl plague virus was able to multiply or cause any alteration in cultures of HeLa cells. Newcastle disease virus, however, as shown by Henle (1954) and by Tyrrell (1955), can be easily propagated in HeLa cells, causing a striking cytopathic effect.

The capacity of fowl plague virus to multiply with cytopathic effect in cultures of human lung does not warrant the conclusion that it is a potential human pathogen. This possibility, however, should be kept in mind, and the fact that no human infections by this virus have been reported may be due to the restricted number of laboratories in which it is handled. In our own laboratory no antibodies against fowl plague virus were found in the blood of workers exposed to risk of accidental infection.

The ability to multiply in human tissues is one more character which fowl plague and Newcastle disease viruses share with others of the group of *Myxovirus* (Andrewes, 1954) in which they are included.

SUMMARY.

Fowl plague and Newcastle disease viruses were successfully propagated in cultures of embryonic human lung.

Newcastle disease virus did not give rise to cytopathic effects throughout the series of 8 passages in these cultures.

Fowl plague virus became cytopathogenic on the third passage and this property was enhanced in successive cultures.

The human-cytopathogenic variant of fowl plague virus did not revert to the parent form after three limit-dilution passages in embryonated eggs.

We are indebted to Mr. M. Young, Biophysics Department, National Institute for Medical Research, for the photomicrographs.

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METABOLIC STUDIES ON SURVIVING HUMAN PROSTATIC TISSUE.

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ENDOCRINE disturbance is believed to play some part in the aetiology of both benign hyperplasia and carcinoma of the prostate, and the successful use of oestrogens in the treatment of prostatic carcinoma makes it desirable to investigate the action of the sex hormones on the metabolism of prostatic tissue. Unfortunately experimental work on both conditions has been hampered because they do not occur commonly in animals. It was decided therefore to study the metabolism of human prostatic tissue removed surgically, a procedure which, although technically difficult, eliminates possible fallacies due to species differences between man and animals.

Previous work on the respiration of human prostatic tissue, using the typical nodules of benign hyperplasia, has been carried out with widely different results by Tolins and Moore (1941), who obtained a Q_{O_2} of 0·19, and Barron and Huggins (1944), who obtained a Q_{O_2} of 1·95, and stated that in dog's prostate the male sex hormones take part in the regulation of the oxidative phase of the carbohydrate metabolism.

Oestrogens have produced squamous metaplasia in the prostates of man and animals (Parkes and Zuckerman, 1935; Moore and McLellan, 1938; Lisco and Biskind, 1941; Sharpey-Schafer and Zuckerman, 1941; Nanson, 1950) and it is probable that certain other histological features which are present in the human prostate at birth (Andrews, 1951) and also in later life are related to the action of hormones. A special study was made of the histology of the tissue used to see if there were any correlation between histological features and respiration.

MATERIAL AND METHODS.

Experiments were performed on 73 prostates which had been removed by radical prostatectomy less than 5 hr. before the experiment began. They were transported in a large volume of Krebs-Ringer phosphate solution in a container surrounded by ice. The prostates were sectioned into large blocks and the typical nodular areas of benign hyperplasia or areas suspected to be carcinoma were removed. From these smaller blocks thin slices were obtained free-hand by the usual method for the experiment; the remainder of each small block was fixed in 10 per cent formol saline for histological study. The blocks were cut and sliced on ice. The slices were placed on filter paper thoroughly moistened with Krebs-Ringer phosphate solution before being transferred to the flasks.

The oxygen uptake was measured by the direct method of Warburg and the experiments were usually allowed to run for 2 or 3 hr. The medium was Krebs-Ringer solution with the addition of 0·1M phosphate at pH 7·2. The tissue was weighed after drying for 2½ hr. at 110°.

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The effect of testosterone propionate and oestrone, either singly or in combination, on the oxygen uptake was studied by adding the hormones to the medium and comparing the Q_{O_2} with that of a control experiment using slices of similar tissue respiring in Krebs-Ringer phosphate solution to which no hormone had been added. The weight of hormone added to each flask was usually 2 mg. but in certain experiments it was varied between 0·2–2 mg. This was dissolved in ether, pipetted into the dry flask and the solvent removed in a stream of air at room temperature. This gave a finely particulate film of hormone distributed evenly over the bottom of the flask and addition of Krebs-Ringer phosphate solution produced a fine suspension. It has been shown by Hayano, Dorfman and Yamada (1950) that this method of hormone addition is satisfactory.

An attempt was made to discover the mode of action of testosterone on the oxidative phase of carbohydrate metabolism by the addition of certain metabolites and inhibitors of the Krebs citric acid cycle to the medium. These were freshly dissolved in Krebs-Ringer phosphate solution to give a concentration of 0·1M and the pH of the solution adjusted. The effect of these metabolites on the Q_{O_2} of prostatic tissue was studied both in the presence and absence of testosterone and the results were compared with suitable control experiments performed on slices of similar tissue.

In the experiments using hormones and metabolites great care was taken to ensure that the tissue slices in the control flask and the hormone or metabolite flask were as closely similar as possible and it was previously ascertained that over the weight range used in the experiments the oxygen uptake was directly proportional to the weight of tissue.

RESULTS.

The Oxygen Uptake of Prostatic Tissue showing Benign Hyperplasia or Carcinoma.

In 54 experiments the Q_{O_2} of the typical nodules or so-called "adenomata" of benign hyperplasia of the prostate was determined. The average Q_{O_2} values and standard deviations obtained were $2{\cdot}23 \pm 0{\cdot}96$, $2{\cdot}30 \pm 0{\cdot}98$, $2{\cdot}73 \pm 1{\cdot}03$ during the first, second and third hours respectively. The total range of all the Q_{O_2} values was 0·65–5·67. The age of the patient had no effect.

Only two cases of carcinoma of the prostate were investigated. The Q_{O_2} values for the 3 hr. were 5·85, 6·27, 5·29 and 4·63, 5·64, 5·64 respectively. The oxygen uptake of carcinoma was therefore significantly greater than that of benign hyperplasia.

Histological correlation.

The range of Q_{O_2} values obtained in benign hyperplasia was very wide and an attempt was made to discover if any histological features were responsible for this variation. This was difficult because the nodule of benign hyperplasia is composed of variable proportions of epithelial acini and stroma, but it was possible to conclude that the oxygen uptake of a nodule depends mainly on the number of epithelial cells, as judged by the number of acini and their degree of cellularity, and also on the secretory activity of the cells, actively secreting cells having a higher oxygen uptake than resting cells. Squamous metaplasia of the prostatic epithelium had no discernible effect on the oxygen uptake.

The Effect of Testosterone on Oxygen Uptake.

In experiments using the nodules of benign hyperplasia testosterone propionate produced a significant fall in oxygen uptake. The results are summarised in Table I.

TABLE I.—*Oxygen Uptake of Prostatic "Adenoma" with and without Added Hormones.*

Series.	Number of tests.	Average Q_{O_2}. 1st hr.	Average Q_{O_2}. 2nd hr.	Standard deviation. 1st hr.	Standard deviation. 2nd hr.	Average Q_{O_2} difference. 1st hr.	Average Q_{O_2} difference. 2nd hr.	Standard error of difference. 1st hr.	Standard error of difference. 2nd hr.
Without testosterone	33	2·25	2·28	0·86	0·91	0·66	0·83	0·2	0·21
With testosterone		1·59	1·45	0·73	0·65				
Without oestrone	21	2·59	2·55	1·1	1·06	0·21	0·24	0·33	0·32
With oestrone		2·38	2·31	1·19	0·96				

Examination of the individual results showed that the degree of depression of oxygen uptake depended on the amount of testosterone available to the epithelial cells. In each experiment of the main series this was a variable figure because, although the amount of testosterone added to the flasks was constant, the weight of the tissue slices and the histological structure of the tissue varied. A special series of experiments was performed in each of which several flasks were set up containing varying weights of testosterone within the range 0·2–2 mg. Tissue from the same prostatic nodule was added to the flasks, an attempt being made to keep the tissue weights as close as possible. From these experiments it was possible to conclude that there is a steady fall in oxygen uptake as the amount of testosterone available to the epithelial cells increases. This proceeds until the oxygen uptake has been reduced by 40–50 per cent, when additional testosterone produces no further fall and the tissue appears to be saturated with hormone.

In the two experiments with carcinoma testosterone also significantly depressed the oxygen uptake.

Testosterone usually produced its effect in the first hour and maintained the depression throughout the experiment. In several experiments, however, the effect was not apparent until the second or third hour. This delay may have been related to the thickness of the tissue slices, but since testosterone was used in the form of the propionate, time may have been required for hydrolysis of this ester before the reactive groups of the hormone became available to the tissue. In some recent experiments, not reported here, we have used pure testosterone and methyltestosterone and there has been no delay in the action of these substances.

The Effect of Oestrone on Oxygen Uptake.

Oestrone produced no constant significant alteration in the oxygen uptake of the nodules of benign hyperplasia. The results are summarised in Table I. Examination of the individual experiments revealed several anomalous results, but it was not possible to draw any conclusions from these.

No experiments were performed with oestrone using carcinomatous tissue.

The Combined Effect of Testosterone and Oestrone on Oxygen Uptake.

In 4 experiments four flasks were set up containing slices of similar tissue. Testosterone 2 mg., oestrone 2 mg. and 2 mg. each of both hormones were added respectively to three flasks; the fourth was a control, the tissue respiring in Krebs-Ringer phosphate only. The results are shown in Table II. The Q_{O_2} values were unfortunately low and the differences small, but when they are

expressed as a percentage of the Q_{O_2} of the control the effect of the hormones is more apparent. In three experiments oestrone produced little effect, but testosterone depressed the oxygen uptake and maintained this depression in the presence of oestrone. In the fourth experiment both oestrone and testosterone produced a similar degree of depression of oxygen uptake and when used together their effects appear additive.

TABLE II.—*Effects of Oestrone and Testosterone, alone and together on Oxygen Uptake of Prostatic "Adenoma".*

Expt. no.	Hormone.	Q_{O_2}. 1st hr.	Q_{O_2}. 2nd hr.	Difference between control and hormone. 1st hr.	Difference between control and hormone. 2nd hr.	Average percentage difference.
1	*Nil* (control)	2·81	2·63	—	—	—
	Oestrone	2·55	2·55	0·26	0·08	6
	Testosterone	1·58	1·31	1·23	1·32	47
	Oestrone + testosterone	1·51	1·06	1·3	1·57	52
2	*Nil* (control)	1·46	1·17	—	—	—
	Oestrone	1·28	1·00	0·18	0.17	13
	Testosterone	0·66	0·66	0·8	0·51	50
	Oestrone + testosterone	0·79	0·79	0·67	0·38	40
3	*Nil* (control)	1·5	1·5	—	—	—
	Oestrone	1·25	1·25	0·25	0.25	16
	Testosterone	1·0	1·0	0·5	0·5	33
	Oestrone + testosterone	1·0	0·64	0·5	0·86	45
4	*Nil* (control)	1·39	1·39	—	—	—
	Oestrone	1·08	1·08	0·31	0·31	22
	Testosterone	1·13	1·00	0·26	0·39	23
	Oestrone + testosterone	0·92	0·53	0·47	0·86	49

The Effect of Metabolites and Inhibitors of the Krebs Citric Acid Cycle on Oxygen Uptake of Prostatic "Adenoma."

The metabolites used were alanine, pyruvate, citrate, glutamate, α-ketoglutarate, succinate and fumarate. Significant results only occurred in the experiments using succinate which increased the oxygen uptake of prostatic "adenoma" by about 40 per cent and also abolished the effect of testosterone. In a special experiment (Table III) the effect of malonate in conjunction with succinate was studied. As was expected, since it inhibits the succinoxidase system, malonate abolished the increased oxygen uptake produced by succinate and yet when acting alone it had no effect.

TABLE III.—*The Effect of Succinate and Malonate on the Oxygen Uptake of Prostatic "Adenoma".*

Added substrate.	Q_{O_2}.
Succinate	1·84
Nil (control)	0·82
Succinate + testosterone	2·02
Testosterone	0·66
Succinate + malonate	0·93
Malonate	0·94

DISCUSSION.

The average Q_{O_2} of prostatic "adenoma" was found to be 2·42 which corresponds fairly closely to the value of 1·95 reported by Barron and Huggins (1944), and although the total range of Q_{O_2} values was wider than that recorded by these authors, this may be due to the fact that our series was larger. In no experiment did we obtain a Q_{O_2} value as low as that published by Tolins and Moore (1941), although many values were not much greater than 0·53, which Barron and Huggins (1944) give as the Q_{O_2} of human connective tissue.

The range of Q_{O_2} values is due to variation in the histological structure of the tissue. Prostatic " adenoma " is not a homogeneous tissue and the oxygen uptake depends mainly on the number of epithelial cells in the nodule. This is not the only factor and the oxygen uptake is highest in those nodules containing actively secreting cells. The high oxygen uptake is related to the process of active secretion only and not to the build-up of secretory granules within the cell.

The oxygen uptake was measured in only two cases of carcinoma of the prostate. In both the Q_{O_2} was higher than that of " adenoma " although the carcinoma values were close to the upper limit of the Q_{O_2} range of " adenoma ". Prostatic carcinoma has a high epithelial cell content, and is therefore a more homogeneous tissue than prostatic " adenoma ". It was considered that the high oxygen uptake could probably be explained by the high cellularity of the tissue and that it did not necessarily indicate any specific property of carcinoma cells.

Testosterone propionate significantly depressed the oxygen uptake of both prostatic " adenoma " and carcinoma. In experiments using prostatic " adenoma " testosterone appeared to act on the epithelial cells alone and the degree of depression of oxygen uptake was proportional to the amount of testosterone available to the epithelial cells until saturation of the tissue was reached when the oxygen uptake had fallen by 40–50 per cent. There was no indication that testosterone acted on a specific phase of the histological epithelial cell cycle.

The action of testosterone on human prostatic " adenoma " resembles its action on rat brain homogenate (Gordan and Elliott, 1947), but is exactly opposite to its supposed action on dog's prostate described by Barron and Huggins (1944) following their experiments with castration and stilboestrol injections in dogs. In their method, however, they made no allowance for the possible action of other ketogenic hormones in the body, such as the adrenal hormones.

In view of the low Q_{O_2} values of the prostatic " adenomata " it would be surprising to find the Krebs citric acid cycle operating completely, and the experiments with succinate and malonate indicate that, although the succinoxidase system is present, succinic acid formation and oxidation is normally low. The failure of testosterone to inhibit succinate oxidation suggests that it acts on the earlier part of the cycle. Barron and Huggins (1946) have shown that human prostatic " adenoma " utilises pyruvate, alanine and glutamate but not citrate and α-ketoglutarate. There would therefore appear to be a block in the cycle between α-ketoglutarate and succinate and it is suggested that testosterone acts on the α-ketoglutaric oxidase system, competing with the keto-acid for the active groups on the enzyme.

The above hypothesis may explain the disappointing results with oestrone since McShan, Meyer and Erway (1947) and Wight and Burk (1949) have shown that oestrogenic compounds inhibit the succinoxidase system in rat tissues. If,

therefore, the oxidation of succinic acid is normally low in prostatic tissue oestrone could not be expected to produce any significant effect on the aerobic carbohydrate metabolism.

SUMMARY.

The Q_{O_2} of human prostatic " adenoma " was measured by the Warburg technique and found to vary between 0·65–5·67 with an average value of 2·42.

In two experiments the Q_{O_2} of prostatic carcinoma was found to be higher than that of " adenoma ".

It was considered that the oxygen uptake of prostatic " adenoma " depended on the number of epithelial cells in the " adenoma " and on the secretory activity of the epithelial cells. It appeared probable that the higher value obtained with carcinoma was due to the larger number of epithelial cells.

Testosterone significantly depressed the oxygen uptake of prostatic " adenoma " and carcinoma. With " adenoma " the depression of oxygen uptake was proportional to the weight of testosterone available to the epithelial cells; saturation of the tissue with hormone occurred when the oxygen uptake was depressed by 40–50 per cent.

Oestrone had no significant effect on the oxygen uptake of prostatic " adenoma " and testosterone depressed the oxygen uptake in the presence of oestrone.

Alanine, pyruvate, citrate, glutamate, α-ketoglutarate and fumarate had no effect on the oxygen uptake of prostatic " adenoma " and did not interfere with the action of testosterone. Succinate considerably increased the oxygen uptake and abolished the depression produced by testosterone. Malonate abolished the increase in oxygen uptake produced by succinate but by itself had no effect on oxygen uptake.

The significance of these findings is discussed in relationship to the possible mode of action of the hormones on the oxidative phase of carbohydrate metabolism of prostatic " adenoma ".

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COMPENSATORY HYPERTROPHY OF THE RAT THYROID AFTER PARTIAL THYROIDECTOMY.

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REMOVAL of a large portion of the thyroid gland is followed by compensatory hypertrophy of the remnant. This observation, first recorded by Wagner (1884) has been confirmed many times and the literature reviewed by Loeb (1919), Marine (1926) and Cameron (1952). The experiments reviewed have not included any precise functional studies, and we have therefore measured the uptake of radioactive iodine (^{131}I) by the residual thyroid at varying time intervals after partial thyroidectomy. The findings were compared with the ^{131}I uptake of the thyroids of sham-operated controls and correlated with gland weight, histology and follicular cell height. We have tried to analyse the mechanism whereby function is restored to determine the time interval taken by the remnant to reach a steady state and to assess the rôle of the pituitary in this process.

MATERIALS AND METHODS.

Rats.—Stock albinos of mixed origin, mostly males of 250–350 g.

Operations.—All were done under ether anaesthesia. Hemithyroidectomy: the left lobe and left half of the isthmus were removed. Three-quarter thyroidectomy: the left lobe, isthmus and lower half of the right lobe were removed. Sham operations: the thyroid gland was exposed under anaesthesia, but not otherwise manipulated, in all of the controls. Hypophysectomy: the basi-sphenoid was exposed through the lateral pharyngeal approach, drilled and the pituitary sucked out. Completeness of the operation was confirmed at necropsy by examination of the pituitary fossa through a lens and by the finding of gross atrophy of the adrenals and gonads. Adrenalectomy: the left adrenal gland was removed through an incision in the back. There was a post-operative mortality and in addition all rats which did not appear to have made a good recovery from the partial thyroidectomy or hypophysectomy were killed. This led to the loss of 20 animals. The survivors only are enumerated in the experiments below.

Techniques.—After killing the rats with coal gas, the thyroids were removed attached to the trachea, fixed 4 hr. in Helly's fluid and washed overnight in running tap water.

Measurement of radioactivity.—After fixation and washing, each thyroid still attached to trachea was suspended at a fixed point in a standard $1\frac{1}{2} \times \frac{3}{4}$ in. (3·6 × 1·8 cm.) screw-capped vial and counted in the multitube Geiger-Müller ring counter described by Veall and Baptista (1954). The count rates given by these thyroids represented organically bound ^{131}I only, since most inorganic iodide had been washed out of the tissues. We confirmed this in a preliminary trial with 6 rats in which organic binding had been prevented with propylthiouracil. Following an injection of ^{131}I most of the radioactivity was found in the fixing and washing fluids. We also confirmed the validity of our method of measuring radioactivity in 6 rats 3 days after injection of ^{131}I, by which time practically all the ^{131}I in the thyroid is organically bound. The fixing and washing fluids were virtually free of ^{131}I, the count rates of the solid

glands corresponded excellently with subsequent measurement of fluid digests of the same thyroid glands.

In vivo counting of thyroid radioactivity was carried out by immobilisation of the rats under ether anaesthesia in the holder and multitube ring counter described by Arnott and Fossey (1952).

Weighing.—After measurement of radioactivity each thyroid was dissected off the trachea, freed of muscle, and weighed to the nearest 0·2 mg. on a torsion balance.

Histological methods.—After weighing, the thyroids were dehydrated in alcohol, cleared in chloroform and embedded in wax so as to be cut horizontally through the level of the isthmus. Sections were cut at 5 μ and stained by haematoxylin and eosin and by the periodic acid Schiff (P.A.S.) method.

Measurement of cell heights.—Done at oil immersion magnification on the P.A.S.-stained sections with a calibrated linear micrometer eyepiece. The eyepiece was swivelled round and every follicle cell measured whose long diameter lay directly over the image of the linear micrometer. The microscope stage was moved field by field so as to cover the sections completely both longitudinally and transversely : 100 cells were measured in each gland.

Hormone preparations.—L-Thyroxine (Glaxo) was freshly prepared as follows : 5 mg. crystalline L-thyroxine was dissolved in 1·0 ml. 0·1 N-NaOH and the solution diluted in 50 ml. saline. ACTH : Armour's acthar gel, 10 Armour units per ml. T.S.H. : Armour's thytropar, freshly dissolved in saline before use.

EXPERIMENTAL.

Changes in Residual Thyroid Tissue at Varying Time Intervals after Partial Thyroidectomy.

In order to measure the response of the thyroid to hemithyroidectomy 7 groups of rats, an average of 20 per group, were treated as follows. The rats in each group were operated upon on the same day, alternate animals being hemithyroidectomised and sham-thyroidectomised, and were kept in the same cage. They were of similar weight. At 7 different time intervals varying from 2 hr. to 120 days after the operation groups of rats were injected intraperitoneally with 15 μc ^{131}I and killed 2 or 3 hr. later. The thyroid or thyroid remnant was removed, fixed, weighed, its radioactivity measured ; the gland was sectioned and the average follicle cell height determined. Additional three-quarter-thyroidectomised rats were added to the 55- and 120-day groups. We chose a small time interval after ^{131}I injection for the measurement of iodine uptake by the thyroid because we thought there would be no significant output of labelled hormone in under 3 hr., even under conditions of a marked increase in thyroid activity.

The *results* are summarised in Table I and the Figure. The mean weight at 5 hr. of 55 per cent and ^{131}I uptake of 61·6 per cent of the controls represented the tendency in our rats of the right lobe to be slightly larger than the left. The residual lobe after hemithyroidectomy did not increase in weight during the 10 days following the operation. The findings at 25, 55 and 120 days of 63, 71 and 64 per cent as compared with 55 per cent at 5 hr. are suggestive, but by no means definite, of a slight weight gain. By 3 days ^{131}I uptake had risen to 86 and at 10 days to 91 per cent and thereafter did not show any statistically significant difference from the uptake of the controls. By 3 days there was a significant increase in mean follicle cell height of the residual lobes to 118 per cent of the controls. The mean cell height increased further to 131 per cent at 6 and 140 per cent at 10 days. Thereafter the cell height showed no further increase but varied from 137 per cent at 25 to 127 per cent at 130 days. The

residual half lobes of the three-quarter-thyroidectomised rats appear to have reached a fairly steady state by 4 months. They weighed 37 per cent of the controls at both 55 and 120 days : the ^{131}I uptake was within normal limits and the mean follicle cell height was 158 and 133 per cent. There was no significant difference in body weight between the controls and partially thyroidectomised rats within any one group. They all gained weight normally. Thus, for example, in the 55-day group the controls weighed 250 $\pm$ 19 g. at the beginning of the experiment and 336 $\pm$ 22 g. when killed ; the hemithyroidectomised 253 $\pm$ 19 g.

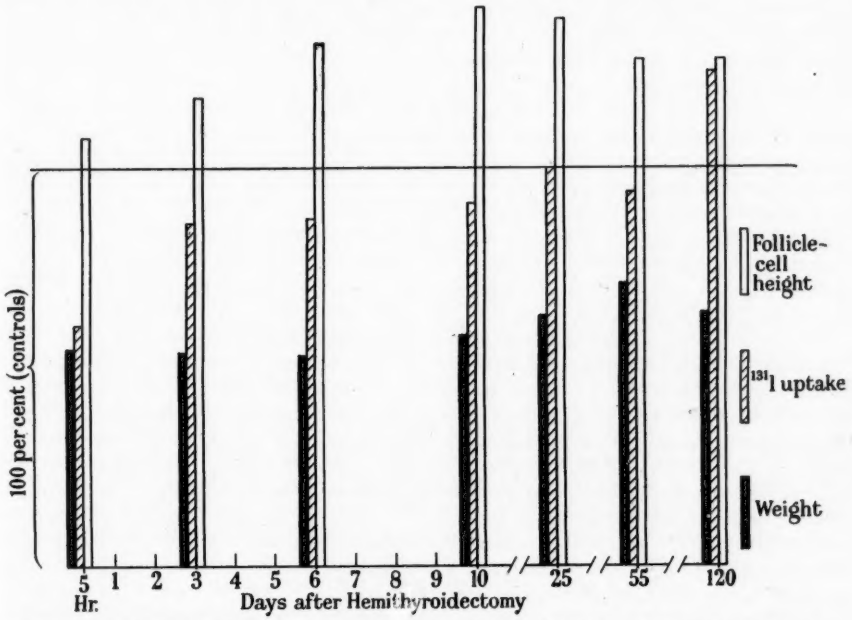

FIGURE.—Weight, ^{131}I uptake and follicle cell height of residual lobe of thyroid after hemithyroidectomy expressed as a percentage of the controls' intact thyroid weight, ^{131}I uptake and follicle cell height.

and 351 $\pm$ 35 g. respectively ; and the three-quarter-thyroidectomised 257 $\pm$ 26 g. and 340 $\pm$ 36 g.

Microscopy showed follicle cell hypertrophy in the residual lobes from the 3rd day onwards. The cytoplasm was more voluminous and less densely eosinophilic and the cells showed an obvious increase in height. A very occasional follicle cell mitosis was observed in the residual lobes on the 3rd, 10th, 25th, 55th and 120th days. The colloid appeared less in quantity, less densely eosinophilic and more vacuolated than that of the controls. The interfollicular capillaries were larger and produced a marked indentation of the follicular cells. These changes were all more advanced in the centrilobular than in the peripheral follicles. There was no apparent increase in the number or size of follicles, although the lobules appeared more loosely packed.

TABLE I.—*Changes in Thyroid Weight, Follicle Cell Height and ^{131}I Uptake at Varying Time Intervals after Partial Thyroidectomy.*

Time interval after partial thyroidectomy.	Group.	Number of rats.	Mean thyroid weight (mg.) + S.D. of mean.	Residual thyroid lobe weight as per cent of controls.	Mean thyroid uptake of ^{131}I in 1000 counts/min. + S.D. of mean.	Residual lobe ^{131}I uptake as per cent of controls.	Mean thyroid follicle cell height (μ) + S.D. of mean.	Residual thyroid lobe follicle cell height as per cent of controls.
5 hours	Controls	10	21·7±2·4		10·6±2·9		6·93±2·41	
	Hemithyroidectomy	10	11·9±1·5	55	6·4±1·5	61	7·49±0·72	108
3 days	Controls	11	30·3±3·9		3·7±0·9		7·34±0·61	
	Hemithyroidectomy	10	16·2±2·0	54	3·2±0·7	86	8·66±0·35	118
6 ,,	Controls	10	23·6±3·1		1·7±0·5		6·88±0·58	
	Hemithyroidectomy	10	12·4±2·1	53	1·5±0·4	87	9·01±1·27	131
10 ,,	Controls	10	24·6±4·3		3·2±1·6		5·93±0·61	
	Hemithyroidectomy	11	14·3±2·3	58	2·9±1·0	91	8·32±0·94	140
25 ,,	Controls	10	27·8±3·8		3·0±0·7		7·07±0·66	
	Hemithyroidectomy	10	17·6±3·0	63	3·0±0·7	100	9·69±0·70	137
55 ,,	Controls	8	26·3±4·7		1·6±0·5		8·30±1·46	
	Hemithyroidectomy	8	18·7±3·7	71	1·5±0·5	94	10·20±1·99	127
	Three-quarter thyroidectomy	8	9·8±3·1	37	1·5±0·5	94	13·15±1·64	158
120 ,,	Controls	9	32·3±3·0		1·6±1·0		7·46±0·82	
	Hemithyroidectomy	9	20·7±3·6	64	2·0±0·9	124	9·45±0·74	127
	Three-quarter thyroidectomy	8	11·9±3·6	37	1·5±0·8	94	9·91±0·72	133

The rats were each injected with 15 μc ^{131}I 2 or 3 hr. before they were killed The radioactivity of the thyroids of any one group was assayed at the same time. The variation in count-rate between groups is due mainly to variation in the time interval of assay after removal of the thyroids.

Rate of Loss of ^{131}I from the Thyroid after Three-quarter Thyroidectomy.

The finding of a maintained increase in follicle cell height in the residual lobe is evidence of an increased stimulation by pituitary thyrotrophic hormone (T.S.H.). We tried to find an objective measure of the associated colloid store. The smaller colloid store in the partially thyroidectomised rats is likely to be associated with a smaller store of thyroid hormone. This would result in a higher ratio of labelled to unlabelled hormone in a tracer experiment. The higher specific radioactivity of the stored hormone would lead to a more rapid loss of ^{131}I from the residual lobe though the total output of hormone, labelled + unlabelled, would be the same as that of the control thyroids. The rate of loss of ^{131}I from the thyroid was measured in the following experiment.

Ten rats three-quarter-thyroidectomised 2 months previously and 11 sham-thyroidectomised rats were injected intraperitoneally with 25 μc ^{131}I. Their thyroid radioactivity was measured *in vivo* under ether anaesthesia at intervals of 12, 24, 36, 60, 84, 102 and 132 hr. after the injection. At each interval the counts/min. registered were recorded as a percentage of the standard dose of 25 μc ^{131}I counted at the same time. The results, plotted on semi-logarithmic paper, showed a steady fall in thyroid radioactivity from 36 hr. onwards (radioactivity ordinate and hours abscissa). The *slope* of this line was measured for each individual rat (fall in height per unit length of abscissa).

Results.—The percentage uptake of ^{131}I into the thyroid varied considerably between the rats. The average was 22·6 per cent (18·3–27·5) at 12 hr. in the controls, was about the same at 24 hr., dropped to 20·1 per cent at 36 hr. and fell steadily to 12·1 per cent (9·6–15·5) at 132 hr. The three-quarter-thyroidectomised groups showed a lower average content of ^{131}I at 12 hr., *i.e.*, 17·4 per cent (13–22·5) which dropped to 15·4 per cent at 36 hr. and then also fell steadily to 7·1 per cent (3·8–10) at 132 hr. The loss of ^{131}I from the thyroid was significantly greater in the three-quarter-thyroidectomised animals. The mean thyroid ^{131}I was 46 per cent of the 36 hr. content at 132 hr. in the three-quarter-thyroidectomised rats and 60 per cent in the controls. This was confirmed by the mean slope of the plotted points : 0·209 against 0·1376. Student's *t*-test gave a significance of $P < 0{\cdot}001$ for both methods of recording the difference in fall of radioactivity. The difference is reduced by the further uptake into the thyroid of ^{131}I released from the metabolism of labelled hormone in the periphery. Owing to the higher specific radioactivity of the thyroid hormone in the partially thyroidectomised rats, more ^{131}I is made available for re-use than in the controls.

Effect of Thyroxine and of Hypophysectomy on Compensatory Hypertrophy of the Thyroid.

Since pituitary T.S.H. is known to increase thyroid follicle cell height and reduce the colloid store, the results (Table I) suggest dependence of the compensatory hypertrophy response on an intact hypothalamic pituitary mechanism. This mechanism is likely to be stimulated to an increased output of T.S.H. following the temporary drop in thyroxine secretion from the hemithyroidectomised thyroid. Though this seems obvious it is nevertheless also possible that some degree of thyroid hypertrophy might be independent of the pituitary.

We therefore designed the following experiment to see if hypophysectomised rats show any degree of compensatory reaction after hemithyroidectomy. We added a group of non-hypophysectomised animals in which T.S.H. output was inhibited by the daily administration of a large dose of thyroxine.

The animals were arranged in four groups. The 22 rats in the first group were injected daily with 16 μg. L-thyroxine subcutaneously. After 3 days 11 of them were hemithyroidectomised and 11 sham-hemithyroidectomised. The thyroxine injections were continued and 24 hr. later they were all given an intraperitoneal injection of 50 μc ^{131}I and killed after 18 hr. The thyroids were fixed, their radioactivity measured, and they were weighed and sectioned. The remaining groups, 15, 18 and 12 rats respectively were all hypophysectomised and half of them hemithyroidectomised and half sham-hemithyroidectomised. In the last group the thyroid operation was done 21 days after the hypophysectomy. Two groups were killed 27 days and one 22 days after the hemithyroidectomy 18 hr. after receiving 50 μc ^{131}I. The thyroids were fixed, their radioactivity measured and they were weighed and sectioned. The interval of 18 hr. ^{131}I uptake rather than 3 hr. was chosen because of the extremely slow iodine uptake which follows hypophysectomy.

The *results* summarised in Table II show no evidence of any gain in weight of the residual lobe after 3 to 4 weeks. They averaged 42–54 per cent of the controls. Nor was there any evidence of any increased function. The mean ^{131}I uptake of the residual lobe compared with the control lobes was 60 per cent in the thyroxine-treated animals and varied from 52–28 per cent in the hypophysectomised groups. (The ^{131}I uptake showed a strikingly larger variation from rat to rat than that previously observed in the intact animals).

Microscopic examination showed no difference between the residual lobes and control thyroids in this experiment. The follicles rich in colloid, were all lined by a remarkably thin epithelium, barely 2 μ in height. Measurement of the height of these remarkably flattened cells was in effect measurement of nuclear thickness. Any difference between residual lobes and controls in this experiment was too small for us to detect. The picture was essentially the same in both the thyroxine-treated and the hypophysectomised groups.

Comparison of the Residual Thyroid and Adrenal after Hemithyroidectomy and Unilateral Adrenalectomy with Control Thyroid and Adrenals in Hypophysectomised Rats Treated with the Same Dose of T.S.H. and ACTH.

The findings in the previous experiment of no functional or morphological evidence of compensatory reaction after thyroidectomy in the absence of T.S.H. confirm the essential rôle of the pituitary in the process of compensatory hypertrophy of the thyroid. Since thyroid tissue is known to inactivate T.S.H. *in vitro* (Rawson, Sterne and Aub, 1942) it is conceivable that the freshly hemithyroidectomised rat might use up circulating T.S.H. at only half the rate of control rats. Thus, a "normal" output of T.S.H. from the pituitary might lead to an increased blood level in the hemithyroidectomised animal which would consequently lead to hypertrophy of the residual lobe. To investigate this possibility we compared the function and histology of the hemithyroidectomised thyroid with that of the whole gland in hypophysectomised rats given daily injections of the same quantity of exogenous T.S.H. Thus, if thyroid tissue *per se* plays a chief rôle in the removal or destruction of T.S.H., the animals

TABLE II.—*Effect of Thyroxine Treatment and of Hypophysectomy on Compensatory Hypertrophy of the Thyroid after Partial Thyroidectomy.*

Treatment.	Group.	Number and sex of rats.	Mean body weight (g.) + S.D. at time of hypophysectomy.	Mean body weight (g.) + S.D. at time of killing.	Mean thyroid weight (mg.) + S.D. of mean.	Residual thyroid lobe weight as per cent of controls.	Mean thyroid uptake of ^{131}I in 1000 counts/min. + S.D. of mean.	Residual thyroid lobe ^{131}I uptake as per cent of controls.
16 μg. L-thyroxine daily for 27 days. Hemithyroidectomy 3 days after commencement of treatment. Killed 24 days later	Thyroxine only	10 M.	—	313±38	22·7±4·3	—	0·89±0·20	—
	Thyroxine and hemithyroidectomy	11 M.	—	325±24	12·1±3·2	53	0·53±0·25	60
Hypophysectomy and hemithyroidectomy on same day. Killed 27 days later	Hypophysectomy only	9 M	283±12	214±10	17·0±2·5	—	2·49±0·84	—
	Hypophysectomy and hemithyroidectomy	6 M.	278±21	208±12	7·6±1·6	45	1·15±0·61	46
Hypophysectomy and hemithyroidectomy on same day. Killed 22 days later	Hypophysectomy only	8 M.	276±18	248±19	14·9±3·7	—	3·72±1·59	—
	Hypophysectomy and hemithyroidectomy	10 M.	278±25	237±21	6·3±1·3	42	1·94±1·32	52
Hypophysectomy followed 21 days later by hemithyroidectomy. Killed 27 days after hemithyroidectomy	Hypophysectomy only	6 F.	243±30	193±21	14·6±2·8	—	8·29±4·43	—
	Hypophysectomy and hemithyroidectomy	6 F.	239±14	193±30	7·9±2·1	54	2·33±1·75	28

The rats were each injected with 50 μc ^{131}I 18 hr. before they were killed.

with only one thyroid lobe should show a greater stimulation of the thyroid tissue than those with two lobes. We considered that the health of these hypophysectomised animals would benefit from the possession of adrenals which were functioning and therefore gave them ACTH in addition to T.S.H. We also extended the experiment by the removal of one adrenal in the hemithyroidectomised rats. This allowed us to study the quantitative response of the adrenal to its pituitary trophic hormone in the same way as the thyroid.

The thyroid and adrenals always atrophy after hypophysectomy. We therefore did a preliminary experiment on 6 hypophysectomised rats using two different doses of T.S.H. and ACTH to find how much hormone must be given daily to maintain a normal thyroid and adrenal weight. This was effected by 0·75 Armour units ACTH and 0·3 U.S.P. units T.S.H. per day. The main experiment was then carried out. Twenty-five rats were hypophysectomised and in 12 of them an additional hemithyroidectomy and removal of the left adrenal was performed. After 3 days and for the ensuing 10 days all the animals were given daily ACTH and T.S.H. All the animals were killed 24 hr. after the last dose of ACTH and T.S.H.: 3 hr. before death they were injected intraperitoneally with 30 μc ^{131}I. The thyroids and adrenals were fixed, weighed and sectioned. The bound ^{131}I content of the thyroids was measured.

The *results*, summarised in Table III, show clearly that the residual thyroid lobe and adrenal gland were not stimulated more than the control glands. The residual thyroid lobe weighed 56 per cent, gave a 59 per cent ^{131}I uptake, and showed a mean cell height of 102 per cent of the controls. The residual adrenal gland weighed 52 per cent of the controls. Thus there is no evidence that the blood levels of T.S.H. and ACTH were higher in the hemithyroidectomised and hemiadrenalectomised animals. The mean follicle cell height of 6 μ compared favourably with the 6–8 μ found in unhypophysectomised controls (Table I) and contrasted with the 2 μ found in the hypophysectomised rats not given T.S.H. (Table II).

DISCUSSION.

Our findings confirm those of previous workers that there is a compensatory hypertrophy of the residual lobe following hemithyroidectomy (Table I). We have shown that there is a restitution of function to normal (Table I), that this

TABLE III.—*Comparison of the Unilateral Thyroid and Adrenal after in Hypophysectomised Rats Treated with the*

Treatment.	Number of rats.	Mean body weight (g.) + S.D. at time of operation.	Mean body weight (g.) + S.D. when killed
Hypophysectomised and killed 14 days later. Given daily parenteral T.S.H. and ACTH from the 3rd to the 14th day.	13	295±21	258±13
Hypophysectomised hemithyroidectomised, hemiadrenalectomised and killed 14 days later. Given daily parenteral T.S.H. and ACTH from the 3rd to the 14th day.	12	296±13	252±18

The daily dosage of T.S.H. was 0·3 U.S.P. units and of ACTH was 0·75 Armour units per rat.

depends on secretion of T.S.H. by the pituitary (Table II) and that it is likely to be due to an increased pituitary output rather than a diminished destruction of T.S.H. (Table III). Following hemi- or three-quarter thyroidectomy the gland is restored to normal function by a maintained cell hypertrophy and associated decreased colloid store and hormone store (Table I). This state was seen as long as 4 months after hemithyroidectomy. Hypothetically, function might have been restored after hemithyroidectomy by a twofold increase in the number of cells. Restitution of the liver after two-thirds hepatectomy in the rat is brought about by an increase of the number of cells in the stump to the number in the whole control liver (Brues, Drury and Brues, 1936). However, we found no obvious increase in the number of follicles, diameter of follicles or number of nuclei per follicle in the residual thyroid lobes after partial thyroidectomy. Nevertheless, although we cannot rule out a slight degree of cell multiplication, we are confident on histological grounds that increase in cell height and not number is the characteristic pattern seen in compensatory hypertrophy after a hemi- or three-quarter thyroidectomy.

The finding of a maintained increase in follicle cell height is evidence of an increase in the level of circulating T.S.H. The thyroid gland is capable of hyperplasia to a ten- or twentyfold increase in its mass and cell number under the stimulus of excessive endogenous T.S.H. produced by dietary iodine deficiency or goitrogens. One must conclude that the rise in T.S.H. output following hemi- or three-quarter thyroidectomy is far below that following thiouracil administration. A relatively small number of hypertrophied cells is probably able to maintain an output of thyroid hormone sufficient to prevent the order of rise of T.S.H. output which obtains in goitrogen experiments. We picture the sequence of events as follows: Hemithyroidectomy is immediately followed by a moderate fall in level of circulating thyroid hormone. This leads to a moderate rise in T.S.H. output by the pituitary which produces a sufficient hypertrophy of the thyroid cells to bring the blood level of thyroxine up to normal. From then onwards there is no extra rise of thyroxine to diminish T.S.H. output nor any drop in thyroxine level to stimulate a further rise in T.S.H. output. Thus the animal is set with a smaller than normal thyroid made up of hypertrophied cells and diminished colloid associated with a raised level of T.S.H. The greater the initial fall in blood thyroxine level, *i.e.*, the greater the mass of thyroid tissue

Hemithyroidectomy and Hemiadrenalectomy with Control Intact Glands Same Dosage of T.S.H. and of ACTH.

Mean thyroid weight (mg.) + S.D. of mean.	Residual thyroid lobe weight as per cent of controls.	Mean thyroid per cent ^{131}I uptake + S.D. of injected ^{131}I.	Mean residual thyroid lobe ^{131}I uptake as per cent of controls.	Mean thyroid follicle cell height (μ) + S.D. of mean.	Mean residual thyroid lobe follicle cell height as per cent of controls.	Mean total adrenal weight (mg.) + S.D of mean.	Residual adrenal weight as per cent of controls.
19·3±3·0	—	3·2 ±0·84	—	6·04±0·71	—	27·9±3·3	—
10·8±2·1	56	1·89±0·66	59	6·17±0·42	102	14·4±1·7	52

The rats were each injected with 30 μc ^{131}I 3 hr. before they were killed.

initially removed, the higher will be the subsequent rise in T.S.H. Following removal of the majority of the gland one would expect a rise in T.S.H. sufficient to produce the formation of a marked increase in number of cells in addition to cell hypertrophy. Bielschowsky (1949) has shown that the goitrogen-induced excessive rise of T.S.H. which in combination with acetylaminofluorene (A.A.F.) produces thyroid tumours can also be induced by subtotal thyroidectomy. Tumours arose in the residual isthmus of rats treated with A.A.F. following operative removal of both lobes, without any thiouracil treatment.

In our experiments there was only a questionable increase in mass of the residual lobe. The residual lobe after hemithyroidectomy increased at the most by about 20 per cent and the residual half lobe after three-quarter thyroidectomy by about 15 per cent (Table I). Histological study suggested that part of this increase might be due to a greater capillary mass. Very occasional mitoses were seen which might represent some degree of hyperplasia. However, they may also indicate that hypertrophied cells are renewed at a greater rate than non-hypertrophied ones. The residual thyroid tissue can markedly increase its cytoplasmic mass with little change in total mass (Table I). This property is dependent on the lability of the colloid store which diminishes in volume *pari passu* with follicle cell hypertrophy. The intrafollicular spaces in goitrogen-treated rats become mere slits. This reserve of space is of cardinal importance in modifying the pattern of compensatory hypertrophy in the thyroid in contrast to the increase in total weight which occurs in the liver, adrenals and kidneys.

We have so far, in our discussion, neglected the possible rôle of anterior pituitary growth hormone, somatotrophin. It is certainly possible that compensatory hypertrophy of the thyroid is potentiated by somatotrophin. One might argue that failure of the thyroid remnant to hypertrophy in the hypophysectomised rats (Table II) was due to the lack of growth hormone rather than lack of T.S.H. However, in Group 1 (Table II) growth hormone was available but the secretion of T.S.H. was totally inhibited by daily thyroxine administration. Thus T.S.H. would appear to be the major stimulus to compensatory hypertrophy of the thyroid.

Our findings can be summed up by Cameron's (1952) definition that " hypertrophy is controlled growth governed by the need to be overcome ". In the rat thyroid, partial removal is followed by a rapid restitution of function effected chiefly by a maintained cell hypertrophy mediated through the pituitary secretion of T.S.H. The thyroid differs from the rat liver in which function is restored by eventual restoration of the cells in number rather than by a permanent increase in the cytoplasmic mass of each cell.

SUMMARY.

The average ^{131}I uptake, weight and follicle cell height of the residual thyroid lobe was compared with controls at varying time intervals after hemi- and three-quarter thyroidectomy in adult rats.

^{131}I uptake fell to about half after hemithyroidectomy but was restored to normal within 10 days. This was associated with follicle cell hypertrophy and diminished colloid storage which was maintained in a steady state for the 4 months studied.

The diminished colloid store was found to be associated with an increased rate of loss of ^{131}I from the residual lobe.

There was no functional increase or compensatory hypertrophy in the residual lobes of rats hypophysectomised or treated with L-thyroxine.

No compensatory hypertrophy of the thyroid and adrenal was found in hypophysectomised rats, half of them hemithyroidectomised and unilaterally adrenalectomised, all of them maintained by daily injections of a fixed dosage of exogenous T.S.H. and ACTH.

On these findings it was considered that after hemi- or three-quarter thyroidectomy in the rat compensatory hypertrophy of the residual lobe is effected by an increased output of endogenous T.S.H. at a level which gives rise to a degree of individual cell hypertrophy and associated diminished colloid storage sufficient to restore total thyroid function to normal. This state is maintained at least as long as 4 months. There is no restitution to a normal total number of follicle cells associated with a normal colloid store.

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MYCOBACTERIUM TUBERCULOSIS: A STUDY OF THE VIRULENCE OF AN ISONIAZID-RESISTANT VARIANT.

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DRUG resistance to isoniazid, as to the other anti-tuberculous agents, has been demonstrated both *in vitro* (Buck and Schnitzer, 1952 ; Knox, King and Woodroffe, 1952 ; Middlebrook, 1952 ; Pansy, Stander and Donovick, 1952 ; Steenken and Wolinsky, 1952 ; Karlson and Ikemi, 1952*a*) and in organisms recovered from the sputum of patients under treatment with this drug (Joiner, MacLean, Pritchard, Anderson, Collard, King and Knox, 1952 ; Steenken, Meade, Wolinsky and Coates, 1952). Subsequently it has been suggested that these isoniazid-resistant variants are less pathogenic than their more sensitive counterparts (Barnett, Bushby and Mitchison, 1953*a* ; Barry, Conalty and Gaffney, 1953 ; Middlebrook and Cohn, 1953). Clinicians, however, are not altogether reassured, for the patients in whom these resistant bacilli are discovered are so often those showing clinical relapse while still under treatment. The methods available in the laboratory for testing mycobacterial virulence at once come under scrutiny.

Classically the guinea-pig is used and when the injection into the thigh of living tubercle bacilli is followed by progressive generalised tuberculosis such an inoculum is regarded as virulent. But whether this finding affords a criterion for the behaviour of the same organisms towards man is uncertain.

Moreover the results obtained experimentally by various investigators into the pathogenicity of isoniazid-resistant variants are somewhat conflicting (Middlebrook and Cohn, 1953 ; Barnett, Bushby and Mitchison, 1953*b* ; Karlson and Ikemi, 1952*b*, 1954 ; Peizer, Widelock and Klein, 1953 ; Steenken and Wolinksy, 1953 ; Bloch, Widelock and Peizer, 1953 ; Morse, Weiser, Kuhns, Fusillo, Dail and Evans, 1954). Thus Barnett *et al.* (1953*b*) could declare that " there is apparently a relation between the degree of isoniazid resistance of strains of tubercle bacilli and their virulence in guinea-pigs and mice " whereas Bloch *et al.* (1953) decided that " there was no correlation between drug susceptibility and pathogenicity for mice on the one hand, and between these two properties and the severity of the disease in the patient from whom the bacteria were isolated, on the other hand ". Significantly, perhaps, these workers used different laboratory animals and different techniques for inoculation. It seemed desirable, then, to examine these aspects rather more critically.

METHODS.

In the experiments to be described, 4 methods of inoculation were employed : mice intravenously ; guinea-pigs intramuscularly in the thigh ; mice by the intra-corneal technique (Rees and Robson, 1950) ; rabbits by the intra-corneal technique (Gardiner, Rees and Robson, 1949).

Cultures.

One culture of *Myco. tuberculosis*, H37Rv was sensitive to 0·01 μg./ml. isoniazid in Dubos albumin medium and the other was a variant in which resistance to the drug had been established by successive sub-culture *in vitro* so that it grew in an environment of 8 μg./ml. isoniazid, but failed to grow in 40 μg./ml.*

For inoculation, a 10-day culture of the sensitive organism was selected from a tube containing no isoniazid and a similar culture of the resistant organism from a tube containing 8 μg./ml. isoniazid.

Animals.

The mice subjected to intravenous inoculation were male albinos with an average weight of 25 g. The guinea-pigs were adults weighing, on an average, 500 g. The rabbits for corneal testing were adults from stock and the mice for corneal testing were male albinos each weighing about 50 g.

Technique.

Each mouse inoculated intravenously received 0·5 ml. of the undiluted 10-day culture, standardised by opacity but not by viable counts. For the guinea-pigs the suspension was standardised to contain approximately 0·5 mg. organisms by wet weight in each 0·5 ml. saline injected into the right thigh. For the mice infected intra-corneally, the original culture was diluted 1/1,000 with 0·05 per cent Tween 80 in physiological saline and for the rabbits the dilution was 1/10,000. The technique then was that customarily followed (Rees and Robson, 1950; Gardiner *et al.*, 1949).

Régime.

Mice intravenously.

One group of 12 mice received the drug-sensitive organism and another 12 the drug-resistant variant. Each of these groups was then subdivided into two further groups, one of which received a standard diet (M.R.C. 41) at the rate of 5 g. per mouse per day, while the other was given the same basic diet but containing 0·3 mg. isoniazid in each 5 g., equivalent to a dose of about 12 mg./kg. body wt. per day.

Any mice which had not succumbed by 6 weeks were then sacrificed. All were examined post mortem for macroscopic evidence of tuberculosis and histological sections were prepared of the lungs, liver, spleen and kidneys.

Guinea-pigs.

Of two groups, each of 5 guinea-pigs, one was inoculated intramuscularly with the drug-sensitive culture and the other with the resistant variant. These groups were then subdivided into 3 and 2 animals respectively, the former receiving a normal diet and no treatment and the latter being given isoniazid at the rate of 10 mg./kg. body wt. daily in two equal subcutaneous injections. This was continued for 8 weeks, after which time any guinea-pigs which had not already died were sacrificed. Post mortem the organs were examined for macroscopic evidence of tuberculosis and sections were prepared of the lungs, livers, spleens, kidneys and inguinal lymph nodes.

Mice intra-corneally.

Of two groups, each of 12 animals, one received the drug-sensitive and the other the drug-resistant inoculum. Further subdivision into "control" and "treated" groups was as before, isoniazid being offered to the latter in the diet at the rate of 12 mg./kg. body wt. daily. Treatment was maintained for 28 days, but observation and recording of the corneal lesions was continued twice weekly for 10 weeks in all. No post-mortem examination was conducted since, in mice, there is no systemic spread of the disease from the cornea.

* By the technique of Middlebrook (1954), this isoniazid-sensitive culture has since been found to be "catalase-positive" *in vitro*, and the isoniazid-resistant culture "catalase-negative."

Rabbits.

Four rabbits were given the drug-sensitive inoculum intra-corneally and another 4 the drug-resistant variant. Two in each group then served as "controls", while the other 2 were given isoniazid for 12 weeks at the rate of 10 mg./kg. body wt. daily in two equal subcutaneous injections. At the end of this time the rabbits were killed and the organs were examined macroscopically, but no sections were prepared.

RESULTS.

Mice intravenously.

Among the mice infected with the isoniazid-sensitive organism and given no drug treatment there was a heavy mortality, and post mortem, evidence of gross tuberculous involvement with, histologically, dense masses of acid-fast bacilli in the lungs particularly and in the liver, kidneys and spleen as well. By contrast, treatment with isoniazid afforded complete protection.

In the two groups infected with the drug-resistant organisms, all mice survived until 6 weeks, but post mortem the lungs were obviously affected with tuberculosis and, histologically, scattered foci of acid-fast bacilli were found in the lungs, liver, spleen and kidneys. Treatment of one of these groups with isoniazid made little difference (see Fig. 1).

Guinea-pigs.

In the group infected with the isoniazid-sensitive culture and given no treatment, 2 out of 3 guinea-pigs died within 3–4 weeks, one from intercurrent infection. Local reactions in the groin were insignificant. Post mortem, there was macroscopic and microscopic evidence of generalised tuberculosis in two of the animals, one of which had died and the other which had survived. Treatment with isoniazid again achieved complete protection.

All the guinea-pigs infected with the drug-resistant culture survived for 8 weeks, and post mortem the only sign of tuberculous disease was a few acid-fast bacilli in the inguinal lymph nodes. This applied to both untreated and treated animals (see Fig. 2).

Mice intra-corneally.

The development and progress of the corneal lesions were of the same order in the three groups of mice that may be referred to as sensitive-untreated, resistant-untreated and resistant-treated respectively. Only in the sensitive-treated group were the lesions suppressed (see Fig. 3).

Rabbits.

In the 2 rabbits infected intra-corneally with the isoniazid-sensitive culture and given no treatment, lesions became established in a characteristic manner. In the rabbits for which the isoniazid-resistant organisms were used, the lesions were slower to develop and never so extensive, and treatment of two of these animals with isoniazid apparently had no effect. However, treatment of the other 2 rabbits given the isoniazid-sensitive infection brought about marked, though not complete, suppression of the corneal disease (see Fig. 4.).

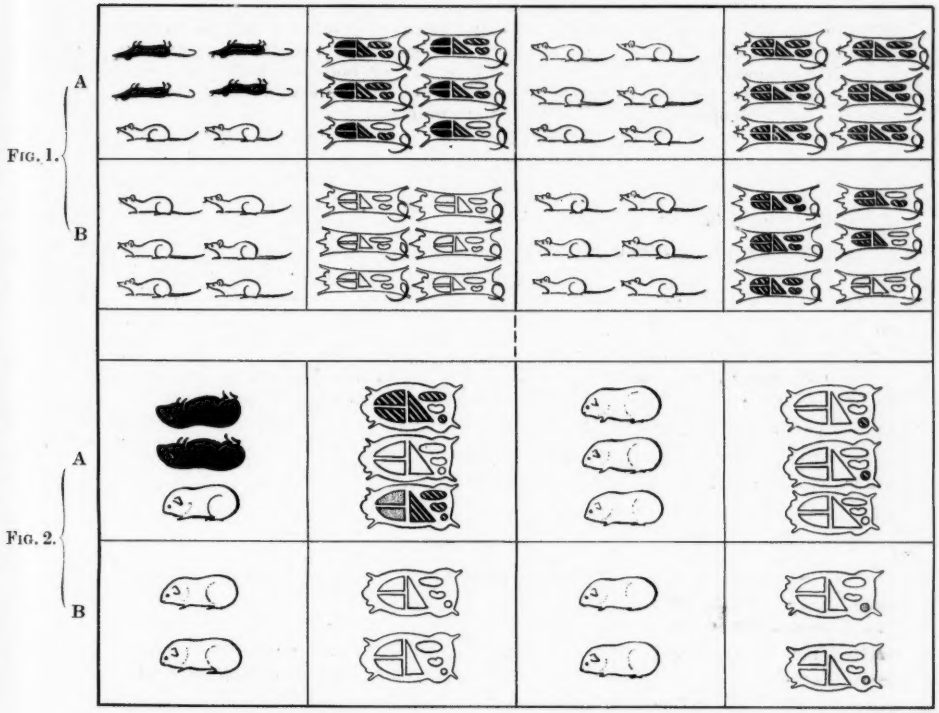

FIG. 1.

FIG. 2.

FIG. 1.—The mortality at 6 weeks and post-mortem findings in mice infected intravenously with H37Rv and a variant in which resistance to isoniazid had been developed *in vitro*. The extent of tuberculosis in the organs is indicated by the degree of shading.

A. Untreated.
B. Treated for 6 weeks with isoniazid (12 mg./kg. daily).

FIG. 2.—The mortality at 8 weeks and post-mortem findings in guinea-pigs infected intra-muscularly with H37Rv and a variant in which resistance to isoniazid had been developed *in vitro*. The extent of tuberculosis in the organs is indicated by the degree of shading.

A. Untreated.
B. Treated for 8 weeks with isoniazid (10 mg./kg. daily).

* In both Figures.

DISCUSSION.

The terms *pathogenicity* and *virulence* are frequently used synonymously, a practice which is evident from the reports quoted in the introduction to this paper. Pagel, Simmonds and Macdonald (1953) have attempted to distinguish between the two terms by defining *pathogenicity* as " the mere power of producing anatomical changes " and *virulence* as " . . . the ability of the tubercle bacillus to multiply locally in the tissues of a susceptible host, and to spread to other parts of the body,

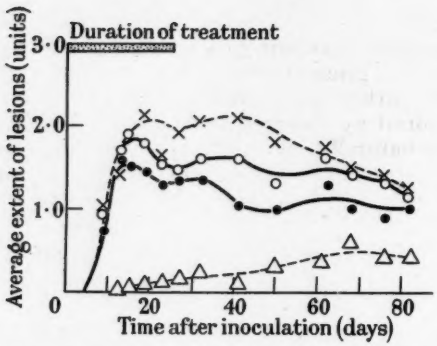

FIG. 3.—Progress of corneal tuberculosis in mice illustrated by curves based on the average size of the corneal lesions (expressed as arbitrary units) for each of the four groups :

isoniazid-sensitive untreated (●———●).
isoniazid-resistant untreated (o———o).
isoniazid-sensitive treated (Δ - - - -Δ) and
isoniazid-resistant treated (× - - - -×).

Treatment of the mice in two of the groups with isoniazid was limited to the first 28 days.

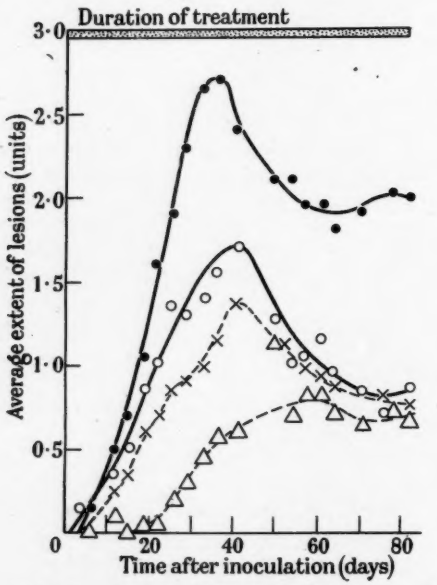

FIG. 4.—Progress of corneal tuberculosis in rabbits illustrated by curves based on the average size of the corneal lesions (expressed as arbitrary units) for each of the four groups : isoniazid-sensitive untreated (●———●), isoniazid-resistant untreated (o———o), isoniazid-sensitive treated (Δ- - - -Δ), and isoniazid-resistant treated ×- - - -×). Treatment of the rabbits in two of the groups with isoniazid extended over the 12 weeks of the experiment.

thus producing progressive lesions at the site of entry and elsewhere ". Miles (1955), on the other hand, suggests that *pathogenicity* should be regarded as a potentiality or " attribute of a species, genus or other grouping of parasites ", whereas *virulence* is " reserved for the pathogenicity of a given stable homogeneous strain of a microbe ", as determined by observed behaviour under certain conditions. This problem of definition naturally arises with the alleged alteration in " virulence " that comes about in tubercle bacilli when they acquire drug-resistance.

Dubos (1955), although emphasising that " the word *virulence* has meaning only in terms of certain highly standardised conditions of test ", maintains that the different strains of mammalian tubercle bacilli exhibit a continuous gradation in this characteristic, from the truly *avirulent*, *e.g.*, H37Ra, which fail to proliferate *in vivo*, through the *attenuated*, *e.g.*, B.C.G., which multiply extensively in host tissues yet bring about only self-limiting lesions, to the truly *virulent*, *e.g.*, H37Rv, capable of causing extensive lesions and progressive fatal disease. The suggestion is now made that the third of these categories should be further subdivided into the *locally virulent*, when the organisms provoke anatomical changes locally and the disease process spreads only by contiguity (*i.e.*, the " pathogenicity " of Pagel *et al.*, 1953) and the *generally virulent*, when the organisms not only cause disease locally but become disseminated throughout the body, by blood-stream or lymphatic spread, to set up generalised disease in other organs.

Experimental corneal tuberculosis, in both the mouse and the rabbit, is a *locally virulent* process. Generalised infection from the eye is unknown in mice (unless they are treated with cortisone) and, with H37Rv, rare and delayed in the rabbit. The present corneal experiments with mice therefore suggest that the isoniazid-sensitive and isoniazid-resistant strains of *Myco. tuberculosis* were of equal *local virulence*. In the rabbit, however, the corneal lesions from the drug-resistant variant neither developed so rapidly nor grew so large as those from the sensitive organisms. A lower concentration of viable organisms may have been responsible, for matching of the two cultures was merely by opacity, with all the fallacies attendant thereto. The progress of the corneal lesion in the mouse is almost independent of the number of organisms injected (Rees and Robson, 1950), but in the rabbit the size of the inoculum exercises a much greater influence.

In the mice infected intravenously the organisms were at once spread throughout the body. The generalised tuberculosis which developed was then due, not so much to an essentially disseminative character of the organisms concerned, *i.e.*, their *general virulence*, but rather to a multiplicity of their *local virulence*. On this basis the drug-sensitive and drug-resistant inocula behaved similarly. Admittedly the density of acid-fast organisms found post mortem in the viscera of the sensitive group of mice was rather more than in the resistant group, a difference emphasised by the relative mortalities in the two groups, but this again might be explained by the unequal content of viable organisms in the two inocula. Pierce, Dubos and Schaefer (1953) have shown that in mice a direct quantitative relation exists between the number of active tubercle bacilli introduced intravenously and the magnitude of the visceral tuberculosis that arises therefrom.

Again, the partial response to treatment among the mice infected with the " resistant " variant points to the cultures which behaved as resistant *in vitro* containing a small proportion of sensitive organisms.

Finally, with the guinea-pigs, a lesion arose in the thigh according to the *local virulence* of the organisms employed. Subsequent dissemination might then

depend on a number of factors, proliferation of the organisms in the initial site, surrounding tissue reactions, macrophage response, lymphatic spread and so on. By the guinea-pig tests, the drug-sensitive culture was *generally virulent*. In 2 of the 3 untreated animals, systemic involvement was such as to cause death. In those given the resistant culture, on the other hand, no more than the regional lymph nodes were affected. Again, *in vivo* resistance was shown to isoniazid treatment where the resistant culture was used as the source of infection.

It is, therefore, tentatively suggested that isoniazid-resistant tubercle bacilli may be of the same *local virulence* as drug-sensitive strains, but that they may be lacking in *general virulence*. Further perusal of the findings on this subject by Karlson and Ikemi (1952*b*, 1954), Peizer *et al.* (1953), Steenken and Wolinsky (1953), Bloch *et al.* (1953) and Morse *et al.* (1954) shows that they are all in accord with this thesis. The results obtained by Middlebrook and Cohn (1953) from guinea-pigs infected intravenously are, however, at variance, but the authors do not explain how they standardised their inocula. And while Barnett *et al.* (1953*b*) generalise about the inverse relationship between drug-resistance and " virulence ", with some justification according to their experiments with guinea-pigs infected in the thigh, the evidence for this which they obtained from mice inoculated intravenously is by no means convincing.

Clinical findings may also support this thesis. Cohen and Glinsky (1954) observed fifty-two cases of pulmonary tuberculosis from which isoniazid-resistant organisms had been recovered. Eighteen of these clearly showed advancing disease. But, to quote the authors, " In most cases, this took the form of extension of existing foci, but sometimes new lesions (bronchogenic spreads) developed. New *extra*-pulmonary lesions did not develop ".

If, indeed, there is a loss of *general virulence* in isoniazid-resistant variants of *Myco. tuberculosis* without, at the same time, any loss of *local virulence*, the underlying mechanism of this difference is still to be explained.

SUMMARY.

In the reports compiled by various workers studying the " virulence " of isoniazid-resistant variants of *Myco. tuberculosis*, the findings lack agreement. Many clinicians are sceptical of the alleged " avirulence " of drug-fast strains.

The " virulence " of isoniazid-resistant cultures of *Myco. tuberculosis* has been compared experimentally with that of their isoniazid-sensitive counterparts by four different techniques, namely, in mice by intravenous injection, in guinea-pigs by intramuscular injection, and in mice and rabbits by intra-corneal inoculation.

The findings lead to a reconsideration of definitions. It is suggested that both isoniazid-sensitive and isoniazid-resistant organisms are *locally virulent*, *i.e.*, capable of setting up local disease spreading by contiguity, but that only isoniazid-sensitive strains are *generally virulent* as well, *i.e.*, capable of setting up disease from a local focus to spread throughout the body of the host.

It is a pleasure to record my indebtedness to Professor J. M. Robson for the facilities he has readily given me and for his continued encouragement and advice, and to Professor R. Knox and Professor G. Payling Wright for their helpful criticism. My thanks are further due to Miss Margaret Woods for her conscientious

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MODIFICATION OF THE REACTION TO VOLE BACILLUS INOCULATION IN THE GUINEA-PIG.

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THE murine strain of *Mycobacterium tuberculosis* (the vole bacillus) has been used for several years as a vaccine to confer increased resistance against subsequent tuberculous infection (Wells, 1949 ; Hall and Wylie, 1952). In the great majority of subjects there has been no serious incidence of local or general reaction. However, as with B.C.G. (Gaisford and Griffiths, 1954 ; Myers, 1954), untoward reactions have occurred following intradermal inoculation. Local lupoid changes have been described (Wylie, Bennett and Swithinbank, 1954). Severe regional lymphadenitis, with abscess formation in some cases, has been observed in a considerable proportion of infants (personal observations), although the incidence of lymphadenitis in older children and adults is very low.

As the intradermal route is preferable for inoculation, a series of experiments has been carried out to attempt to modify factors affecting spread of organisms from the site of inoculation. From these experiments it was hoped that a means of reducing the incidence of troublesome clinical lesions in the " vaccinated " human might be evolved.

MATERIALS AND METHODS.

Animals.

Albino guinea-pigs of identical strain and similar weights were used throughout.

Procedures.

Twenty animals were segregated into 4 groups, eight in the first, and four each in the other 3. The animals in the first group (A) each received a single injection of 0·25 mg. of live vole bacilli (murine strain OV121) suspended in 0·5 ml. isotonic saline into the right gastrocnemius muscle. All the injections were made by one experimenter throughout, and attempts were made to standardise the site and depth of the injection. A second group (B) received 0·25 mg. of the same organism made up in 0·5 ml. of saline plus 250 units of Hyalase (Allen and Hanbury brand hyaluronidase) in 0·25 ml. saline at the same site. A third group (C) was similarly injected with 0·25 mg. of the organism in 0·5 ml. saline plus 0·25 ml. fresh guinea-pig blood, obtained immediately beforehand by cardiac puncture of one of the animals used.

A fourth group (D) received 0·25 ml. of a heavy suspension of the same organism, previously killed by autoclaving at 115° for 30 min., injected into the left gastrocnemius muscle 7 weeks before the other groups were inoculated. After 52 days, when all animals were strongly positive to tuberculin 1/100, live vole bacilli were injected, as in Group A, into the right gastrocnemius muscle.

The rates of Mantoux conversion were estimated by frequent skin testing with increasing dilutions of Old Tuberculin. One animal from each group was killed and examined at 2

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weeks and 4 weeks respectively after the live vole bacillus inoculations and the survivors were all killed and examined 6 weeks after inoculation. Examination embraced macroscopic and histological changes and bacteriology.

RESULTS.

In order to determine the extent of lesions produced by the vole bacillus both locally and generally the four groups were examined as follows.

The local lesion was inspected at intervals during life and at death was examined histologically and material was obtained for culture. Likewise the following viscera were specifically examined in the same way. The ipsi-lateral regional lymph node ; the contra-lateral regional lymph node ; both internal iliac glands ; the para-aortic glands ; the spleen ; the pulmonary hilar glands.

Attempts were made in each case to recover the injected vole bacilli by culture, but the vole bacillus is extremely dysgonic on all the usual laboratory media used for the culture of tubercle bacilli and is, moreover, extremely difficult to grow from small inocula. The inoculum of even the most obviously infected tissues and organs is very small, and, as the number of bacilli present in the more remote lesions declines, we were unable to recover the organisms, in many cases when they were almost certainly present. It was therefore felt that the presence of a typically tuberculous tissue reaction was a more reliable guide to the presence of the vole bacilli than either direct microscopy or culture. As the vole bacillus was the only organism inoculated there was no reasonable doubt that the tissue reaction produced was caused by this organism alone. The results of the bacteriological investigation are given in Table I, which comprises the results obtained in all the animals of each group.

TABLE I.—*Positive Cultures of Vole Bacillus Obtained from Viscera Examined.*

	Group A.	Group B.	Group C.	Group D.
Local lesion	+	+	+	−
Ipsi-lateral lymph node	+	+	+	−
Iliac glands	−	+	−	−
Spleen	−	+	−	−

Summary of Findings in Group A.

After two weeks.

Local lesion.—Inflammation of area of inoculation, slight palpable enlargement of regional lymph gland ; no gross changes observed elsewhere.

Histology.—Typical tubercles were found in the local lesion and regional gland on the ipsi-lateral side only. Acid-fast bacilli were seen in both sites.

Culture.—The local lesion alone yielded a growth of vole bacilli.

After four weeks.

Local lesion.—Skin breakdown over vaccinated area with sparse discharge of caseous material on pressure.

Ipsi-lateral regional node.—Considerable enlargement with early caseating necrosis.

Contra-lateral node.—Macroscopically normal.

Internal iliac glands.—Both enlarged, ipsi-lateral more than contra-lateral, but no necrosis. No other gross change.

Histology.—Evidence of the presence of tuberculous infection was found in the local lesion, the ipsi-lateral regional node, both internal iliac glands and both para-aortic glands. Two solitary tubercles were found in the spleen.

Culture.—Organisms were recovered only from the local lesion and the adjacent lymph gland, although, microscopically, acid-alcohol-fast bacilli were seen in one iliac gland.

After six weeks.

The process seemed to have regressed. There was no macroscopic evidence of involvement beyond the left internal iliac gland and histologically the para-aortic glands and the spleen showed no evidence of tuberculous lesions.

Summary of Findings in Group B.

After two weeks.

Local lesion.—Well-developed but no evidence of skin breakdown.

Ipsi-lateral regional gland.—Palpably enlarged and showed evidence of early caseation.

Iliac glands and para-aortic group.—Enlarged. The spleen also was enlarged.

Hilar glands.—Slightly more prominent than normal.

Histology.—Tubercles were found in the local lesion, regional lymph node, para-aortic group and spleen.

Culture.—The organism was recovered only from the local lesion and the regional node.

After four weeks.

The process had extended and the remaining animals seemed listless and their pelts lack-lustre. Caseation, however, was noticeably absent except for the local lesion, which was less than in the control animals. Evidence of infection was present up as far as the pulmonary hilar glands. The contra-lateral inguinal lymph gland did not appear to be involved. Successful culture was achieved from the para-aortic glands and spleen in addition to the local lesion, ipsi-lateral regional node and iliac glands.

After six weeks.

No significant change was observed.

Summary of Findings in Group C.

The findings in this group differed in no significant respect from Group A. It was hoped that the use of blood in the suspension of the organism might have a localising effect on the lesion but no histological evidence of this was obtained.

Summary of Findings in Group D.

These received 0·25 mg. live vole bacillus 7 weeks after inoculation with dead vole bacilli. The animals were tuberculin-sensitive before they were inoculated

with live vole bacilli. The differences both macroscopically and microscopically, in this group, when compared with Groups A, B and C, were striking.

After 2 weeks there was a small localised area of induration over the inoculation site with only the slightest enlargement of the regional gland. Histologically, there was no evidence of involvement beyond these two sites. Vole bacilli were not isolated from any site. After 4 and 6 weeks the local lesion was entirely resolved. While there was still a small residue of tissue reaction after 4 weeks to be found in the ipsi-lateral lymph node, by 6 weeks all trace of reaction had disappeared except for local fibrosis. In no case was the vole bacillus isolated in culture.

The maximum involvement of each organ examined for all 4 groups is summarised in Table II.

TABLE II.—*Maximum Tuberculous Lesions in Each Group.*

	Group A. Live vole alone.	Group B. Live vole and hyalase.	Group C. Live vole and whole blood	Group D. Dead vole and live vole.
Local lesion	++++	+++	++++	++
Ipsi-lateral lymph node	+++	+++	+++	+
Iliac glands	+	++	+	−
Para-aortic glands	+	++	+	−
Spleen	+	++	+	−
Pulmonary hilar glands	−	+	−	−
Contra-lateral inguinal lymph node	−	−	−	−

The results obtained in this initial experiment suggested that inoculation with dead vole bacillus before inoculation with the living might reduce the incidence of disseminated lesions and the severity of the local reaction. The other techniques used did not appear favourably to modify the results of inoculation with the live vole organism alone.

It was necessary, therefore, to determine whether the time of survival of guinea-pigs infected with a lethal dose of a virulent *Myco. tuberculosis* was different in two groups of guinea-pigs, the first being previously inoculated with OV121, the second with OV121 preceded by a preparatory inoculation of a killed OV121. Albino guinea-pigs of identical strain and similar weights to those in the first experiment were used.

Additional Experiments.

Group X.

Twelve animals were treated in a similar way to those in Group D of the initial experiment, the time between inoculation of the dead and living organisms being 31 days, the animals reacting to 1/100 Old Tuberculin at 20 days after inoculation of the dead organism.

Group Y.

A further 12 animals received the live vole organism (OV121) only, at the same time as Group X, and all 24 animals received 0·25 ml. of a heavy suspension of *Myco. tuberculosis* bovine (Branch) into the groin 8 days later. Each animal was examined at death and in all but one there was clear evidence of an overwhelming tuberculous infection.

The mean survival time in Group X (excluding one animal in which no evidence of tuberculous infection was found at death, 43 days after bovine inoculation) was 89 days, the range being 65–124 and in Group Y 79 days, with a range of 56–109.

There was no control group in this experiment since it has been repeatedly established that, using the identical bovine organism, the mean survival time of the guinea-pig is 55 days (Wells and Wylie, 1952).

SUMMARY AND CONCLUSIONS.

It appears from these experiments that tuberculin sensitivity induced in the guinea-pig by inoculation of a dead mycobacterium limits the dissemination of similarly inoculated live vole organisms. There is no evidence that immunity conferred in this way in the guinea-pig is less effective than that following live vole inoculation alone; on the contrary, the tendency is for resistance to be slightly increased.

Since this involves an essentially practical issue, we feel that trial of this method in the human subject is now indicated and that there are apparently no contra-indications to it.

We are grateful to Mr. N. Smith of the Sir William Dunn School of Pathology for providing the cultures.

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SURVEY OF PAPERS.

ZAIDI, HARRISON, KING and MITCHISON describe three series of experiments in which coal-mine dust together with tubercle bacilli of different degrees of virulence was introduced into the lungs of guinea-pigs.

1. Animals previously inoculated and thus sensitised to tuberculin with B.C.G. were injected intratracheally with B.C.G. and dust, and with each separately. The lesions caused by either alone regressed completely: the combination produced slight fibrosis (p. 539).

2. In these experiments an isoniazid-resistant strain of tubercle bacilli of moderate virulence replaced B.C.G. The bacilli alone produced caseating lesions which shrank and calcified. Combined with dust, this organism caused extensive cavitation and death (p. 545).

3. The strain of tubercle bacilli used in these experiments had a greater degree of resistance to isoniazid and a lower degree of virulence. Bacilli alone caused exudative lesions which disappeared almost completely. Combined with dust, they caused massive fibrosis. Observations are also reported on an overgrowth of bronchial epithelium in these animals, and on the survival of this strain of bacilli in the lungs (p. 553).

ROBSON and DIDCOCK describe a method by which the development of tuberculous lesions in the cornea of mice may be followed, using phase-contrast microscopy for the examination of excised corneas. They suggest that the method may be useful in the investigation of some aspects of tuberculous and other infections, and for studying the action of drugs (p. 560).

SLADE, COBURN and SIMICH have exposed mice to an aerial suspension of streptococci containing a radio-active element, and determined the numbers of cocci inhaled by isotope counts on the ashed corpse. These numbers were remarkably constant with different strains of streptococci, and show that the dose of bacteria cannot be a factor in determining whether infection is established by this route. Observations are also reported on the uptake of ^{65}Zn, ^{59}Fe and ^{32}P by streptococci, and on the use of sonic oscillation for disrupting chains and clumps of cocci to obtain uniform distribution (p. 566).

CLARINGBOLD and SOBEY have shown that myxoma virus can be satisfactorily assayed by the measurement of lesion diameters in rabbit skin using multiple intradermal inoculations of different dilutions of unknown and standard preparations. Multiplication of the virus in the suckling mouse brain could be demonstrated by this method (p. 573).

WEINBREN describes a series of operations on the liver of the rat, the results of which cast doubt on the widely held belief that liver regeneration after partial hepatectomy depends on an intact portal blood supply. The essential difference between his experiments and those of others was that deprivation of portal blood was achieved by tying the branch of the portal vein supplying the right lobe. This did not prevent an increase in size of this lobe itself, and of its individual cells and lobules, with numerous mitoses, after removal of the anterior lobes (p. 583).

HARRIS and BARCLAY describe a coverslip chamber which can be completely filled with medium, kept stirred by a steel wire rotated magnetically, and furnished with two electrodes by means of which the oxygen tension of the medium can be determined electrically. The cells in the chamber can be observed microscopically and counted. By means of this device the rate of oxygen consumption of macrophages was determined; it was found to be independent of the oxygen tension. In the absence of oxygen these cells remained viable and motile, and their consumption of glucose was increased (p. 592).

NORLIN recounts his experiences in attempting to purify diphtheria toxin and points out that as it is a labile substance, ultrafiltration is preferable to vacuum evaporation, and dialysis and freeze drying need special care to avoid acidification and loss of flocculating activity. The difference in specific activity between older preparations and more recent ones is discussed (p. 599).

DONNA CHAPRONIERE and PEREIRA have propagated the viruses of Newcastle disease and fowl plague in roller tube cultures of human embryonic lung. Only fowl plague virus was cytopathogenic for human fibroblasts and this property became evident on the third passage and persisted without change after three transfers at limit-dilution in embryonated eggs (p. 607).

ANDREWS and TAYLOR have determined the oxygen uptake of slices of excised human prostates in the Warburg apparatus. It varied with the cellularity of the gland. Testosterone depressed it, and oestrone had no effect. Of some metabolites tested, succinate increased oxygen uptake and antagonised the effect of testosterone, and malonate, although inactive alone, counteracted the effect of succinate (p. 611).

LOGOTHETOPOULOS and DONIACH have made observations on the weight, follicle cell height and iodine uptake of the remaining thyroid gland in rats after removal of one half to three quarters of the gland. Iodine uptake was restored to normal in ten days, the cells increasing in size and colloid storage diminishing. This compensatory hypertrophy did not occur in hypophysectomised rats, and on this and other grounds it is concluded this hypertrophy is produced by increased output of thyroid-stimulating hormone from the pituitary (p. 617).

GOULDING has compared the virulence of isoniazid-resistant tubercle bacilli with that of sensitive organisms, using mice, guinea-pigs and rabbits. He suggests that both isoniazid-resistant and sensitive strains are "locally virulent," but only isoniazid-sensitive strains are "generally virulent" and capable of causing generalised disease (p. 628).

TAYLOR, WYLIE and VOLLUM have found that prior inoculation of guinea-pigs with dead vole bacilli reduces the incidence of disseminated lesions and the severity of local reactions following inoculation with a living strain. This procedure does not impair the guinea-pig's immunity to tuberculosis and they suggest that it should be tried in man (p. 636)

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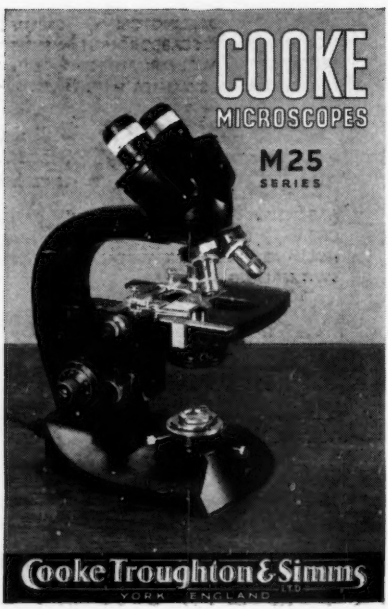

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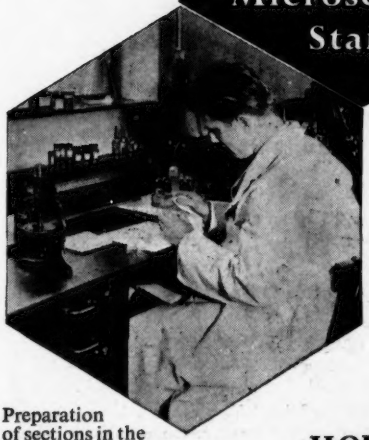

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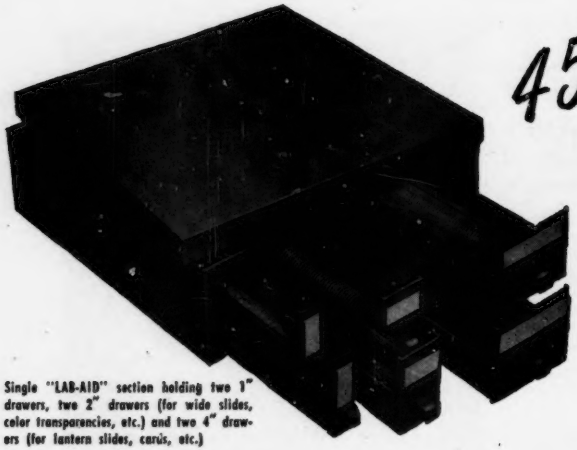

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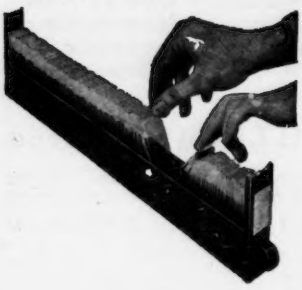

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